

HANDBOOK OF TOXICOLOGY

Third Edition

Edited by

Michael J. Derelanko
Carol S. Auletta



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The third edition of the Handbook of Toxicology is dedicated to the memory of

Dr. Mannfred Hollinger

Dr. Paul E. Newton

Dr. John C. Peckham

Dr. Richard M. Hoar

The contributions of these individuals to the first and second editions of the Handbook of Toxicology appear all or in part in this edition.

Their professionalism, knowledge, and expertise are truly missed.

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Preface to the Third Edition

Nearly 20 years have passed since the first edition of the *Handbook of Toxicology* was published. The purpose of the first edition was to provide information in a consolidated reference source that could not always be easily found and retrieved at that time. The World Wide Web was in its infancy and of little value then. When the second edition went to press, Google had been founded just a few years before and the Internet was becoming more widely used for seeking out the type of information presented in the handbook. Today, the Internet has become a valuable resource to the point that we initially questioned the necessity of a third edition paper reference book. However, many encouraged us to proceed with this edition as they still find a book of this type to be useful for quickly locating information that otherwise requires one to delve through many Internet search “hits” that may be outdated or not relevant. We found this to be the case when conducting searches for various topics to update items from the second edition of the handbook. It is often difficult to determine if information is current or even reliable on the Internet. While new or updated information can be found in any Internet search, one needs to sort through a significant amount of older material that never seems to disappear, making locating pertinent information an increasingly difficult task. Perhaps the pendulum has begun to swing the other way with regard to Internet information retrieval. Nonetheless, the Internet provides a valuable source, especially with regard to frequently changing regulatory requirements.

There are some types of information such as ranges for physiological parameters that maintain their usefulness for many years. Other information such as regulatory guidelines is often updated and modified, in some cases so frequently that it is outdated before the drafts of these books make it to print. In this regard, some information from the earlier editions of the handbook has been reprinted unchanged, other information has been updated where possible, and information that generally changes too frequently to be relevant for a reference book of this type has been removed. In some cases, we have provided updated material for regulatory and other information that tends to change less frequently and have retained older, possibly outdated material (e.g., hazard classifications) for historical purposes to provide the reader with a

source of information that may be needed to understand and interpret older reports and publications and to allow comparison with current practices. As always, the reader is advised to verify that any information provided in this handbook is still relevant for his or her current situation and needs.

All chapters from the second edition of the *Handbook of Toxicology* were reviewed in selecting material for the third edition. The histopathology, clinical pathology, and renal toxicology chapters have been reprinted unchanged. Most of the other previous chapters received minor to major updates. Chapters covering immunotoxicology, endocrine toxicology, and reproductive and developmental toxicology have been completely rewritten to provide a fresh perspective on these topics. Information dealing with inhalation toxicology, neurotoxicology, and regulatory toxicology that was presented in the second edition as several chapters was updated and consolidated into single chapters for each specialty in the third edition. New chapters dealing specifically with chemical and pharmaceutical toxicology, juvenile toxicology, and safety pharmacology have been added. A separate glossary with toxicological terms presented both alphabetically and by toxicological subspecialty has been added. The contributors are experts in the fields of toxicology that they submitted information on. They were asked to provide fundamental and applied information that they felt would be useful to individuals having either a basic or advanced knowledge in the various fields of toxicology covered in this book.

As with the first two editions of the handbook, much of the information provided in the third edition has been previously published elsewhere by others. Considerable effort was made to obtain this information from reliable sources. However, the editors and contributors cannot attest to its accuracy and completeness and, therefore, cannot assume any liability of any kind resulting from the use or reliance on the information provided in this handbook. Mention of vendors, trade names, or commercial products does not constitute an endorsement or recommendation for use.

Michael J. Derelanko
Carol S. Auletta

Preface to the Second Edition

It has been approximately ten years since we began compiling information for the first edition of the *CRC Handbook of Toxicology*. In reviewing the material contained in the first edition, it was apparent that information such as values for physiological parameters, substance toxicity, and information related to fundamental toxicology principles and practices remain virtually timeless. On the other hand, information on such topics as regulatory requirements and guidelines, contract laboratories, and contact information such as phone numbers and addresses clearly needed updating. Moreover, although information for most areas of toxicology was included in the first edition, coverage of some toxicology specialties was clearly missing.

In this respect, the *CRC Handbook of Toxicology, Second Edition* has been extensively updated and expanded. Nearly all of the original chapters from the first edition have been updated, with several receiving extensive revision. Additionally, coverage of inhalation toxicology, neurotoxicology, and histopathology has been expanded. Several new regulatory chapters dealing with pesticides, medical devices, consumer products, and worldwide notification of new chemicals have been added. Areas of toxicology missing from the first edition such as ecotoxicology and *in vitro* toxicology are now covered. Also included is a new chapter providing an extensive overview of the toxicology of metals.

Since the publication of the first edition, environmental and endocrine toxicology and children's health have become major issues that will clearly impact the field of toxicology in the future. To provide some basic information on these topics, two chapters on basic male and female endocrinology and toxicology have been included and tables have been added to the risk assessment chapter that provide information on differences in physiological and biochemical parameters between children and adults. When the first edition went to print, the Internet was in its infancy but has now become an important information-gathering tool for toxicologists. In the

second edition, the authors were asked where possible to reference websites they consider sources of valuable information for their fields of expertise.

The *CRC Handbook of Toxicology* contains a considerable amount of reference information. However, because of the size of the handbook and the number of tables and figures it contains, some users of the first edition reported it was not always easy to identify and locate specific information quickly. As a search aid for the second edition, headings have been added at the top of each page identifying the chapter topics. Also we included pages at the end of some of the chapters to provide additional information closely related to the subject matter of the chapter. Constructive comments on how future editions of the *CRC Handbook of Toxicology* can be improved are welcome.

The number of chapters in the second edition has increased from the original 22 to 33 with over 200 new tables and figures added. It is said, "A picture is worth a thousand words." Thus, as in the first edition, text has been kept to a minimum where possible and practical reference information is provided in tables and figures that are useful to practicing toxicologists in the chemical and pharmaceutical industries, contract laboratories, regulatory agencies, and academia. As before, much of the information provided has been previously published elsewhere. Although considerable effort was made to obtain the information from reliable sources, the editors and contributors cannot attest to its accuracy and completeness and, therefore, cannot assume any liability of any kind resulting from the use or reliance on the information provided in this handbook. Mention of vendors, trade names, or commercial products does not constitute an endorsement or recommendation for use.

Michael J. Derelanko
Mannfred A. Hollinger (Deceased)

Preface to the First Edition

Toxicologists working in the laboratory or office rely on a large information base to design, conduct, and interpret toxicology studies and to perform risk assessments. Diverse information such as normal hematology and clinical chemistry values, reproductive indices, physiological parameters, animal housing requirements, toxicity classifications, and regulatory requirements accumulated during a toxicologist's career are generally scattered in file cabinets and on office shelves. Although practicing toxicologists can usually locate information related to their own areas of expertise with minimal effort, obtaining reference information in less familiar areas of toxicology may require considerably more effort, possibly involving a trip to the library or a phone call to a colleague. A single basic reference source of toxicological information has not been previously available. We have attempted to fill this void with this publication.

Our goal was to produce a reference book containing practical reference information useful to practicing toxicologists in the chemical and pharmaceutical industries, contract laboratories, regulatory agencies, and academia. Contributors were asked to compile reference material for their own areas of expertise, which would be of value to both experts and students. The task seemed easier in concept than it proved to be in reality. It quickly became evident that limits had to be

placed on the amount and detail of information included to allow for publication in a reasonable time frame. Although information for most areas of toxicology is presented, coverage of some areas is clearly missing. We encourage and welcome constructive comments on improving the information provided as well as suggestions for additional material that could be included in possible future editions of this handbook.

We have designed the handbook to allow basic reference information to be located quickly. Each chapter begins with an outline of its contents. Where possible, text was purposely kept to a minimum. This book is intended only to be a basic reference source. The user requiring more detailed discussion should consult the sources cited. Much of the information provided has been previously published elsewhere. The editors and contributors cannot attest to the accuracy and completeness of such and, therefore, cannot assume any liability of any kind resulting from the use or reliance on the information presented in this handbook. Mention of vendors, trade names, or commercial products does not constitute endorsement or recommendation for use.

Michael J. Derelanko
Mannfred A. Hollinger (Deceased)

Helpful Tips for Using This Handbook

The *Handbook of Toxicology* has been designed to allow the working toxicologist to locate basic toxicological information quickly. Where possible, text has been kept to a minimum with much of the information provided in tables and figures. The information is organized into chapters that deal with various areas of toxicology. Each chapter begins with a detailed listing of all of the major topics. Headings are provided on the top of each page, identifying the chapter topic to allow quick location of the subject matter. Because of the large and varied amount of information in the handbook, the user seeking a specific type of information may not always find reference to it in the index. It is recommended that a user

seeking, for example, information on reproductive parameters for various species, use the detailed table of contents at the beginning of the handbook to locate the chapter on developmental and reproductive toxicology, and scan the contents listing. The user will quickly find that Table 10.5 provides the desired information. Similarly, cage requirements for rats can be quickly found in Table 1.17 of Chapter 1 by locating the chapter on laboratory animal management in the table of contents and scanning its contents listing. The user is cautioned that some information contained in the handbook may change over time, particularly as relates to regulatory requirements and guidelines, addresses, and phone numbers.

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We extend a special thanks to the authors and publishers who graciously allowed the reprinting of many of the tables and figures in the handbook.

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Dr. Derelanko has authored numerous papers in experimental hematology, gastrointestinal pharmacology, and toxicology. He has been actively involved in educating the public about toxicology, particularly at the middle school level. He has delivered invited lectures on this subject at national meetings. In 2003, Dr. Derelanko received the Society of Toxicology Award for Contributions to the Public Awareness of the Importance of Animals in Toxicology Research. He is coeditor, along with Dr. Manfred A. Hollinger, of the first and second editions of the *CRC Handbook of Toxicology* (published by CRC Press, Inc., in 1995 and by CRC Press, LLC, in 2002, respectively) and author of the first and second editions of *The Toxicologist's Pocket Handbook* (published

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1 Laboratory Animal Management

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INTRODUCTION

The use of live animals continues to be an important and necessary component of research activities worldwide. To ensure the ethical and humane treatment of animals, scientists must possess a sound understanding of appropriate animal husbandry practices and must be knowledgeable of those variables which may impact and potentially confound experimental procedures and results.

The purpose of this chapter is to provide the scientist with a reference source covering many of the fundamental aspects of proper laboratory animal management for species commonly utilized in toxicological research. In keeping with the desired format of this book, the information provided herein is presented in a concise fashion to allow a broad coverage of animal husbandry topics and related information. For more detailed information, the reader is

referred to the reference materials identified in individual sections, and at the end of this chapter.

ANIMAL HUSBANDRY

Animal husbandry may be simply defined as the methods used in the care and maintenance of animals. In a larger sense, however, animal husbandry encompasses all aspects of appropriate care, treatment, and management for a given species, including circadian rhythm, life span, environmental limits, breeding and reproductive patterns, nutritional and social requirements, environmental enrichment, and macro- and microenvironmental necessities. Of course, each species has its own unique peculiarities that are essential to its well-being. The scientist must be knowledgeable of these characteristics and of the various regulatory guidelines and policies that govern the use of animals in research.

REGULATIONS AND GUIDELINES

Over the years, the United States and other countries have developed various federal mandates and statutes designed to protect animals from illicit commerce and use. The first such federal statute in the United States was the Pet Protection Act of 1966. This act became the forerunner of what is now called the Animal Welfare Act (AWA).

ANIMAL WELFARE ACT

The AWA¹ refers to the Act of August 24, 1966 (P.L. 89-544), as amended by the Acts of December 24, 1970 (P.L. 91-579), April 22, 1976 (P.L. 94-279), December 23, 1985 (P.L. 99-198), November 28, 1990 (P.L. 101-624), January 23, 2002 (P.L. 107-171), May 3, 2007 (P.L. 110-22), and June 18, 2008 (P.L. 110-246). The various provisions of the AWA are designed to ensure that animals used in research, for exhibition, or as pets receive humane care and treatment. The AWA also regulates the transport, purchase, sale, housing, care, treatment, and handling of such animals. The standards set forth by the AWA are considered absolute minimal standards to which people who handle animals must adhere. According to the AWA, “animal” is defined as “any live or dead dog, cat, nonhuman primate, guinea pig, hamster, rabbit, or any other warm-blooded animal, which is being used or is intended for use for research, teaching, testing, experimentation, exhibition, or as a pet.” The term dog means “all dogs including those used for hunting, security, or breeding purposes.”

Regulatory authority under the AWA is implemented by the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture. Rules and regulations pertaining to implementation of the law are provided in the Code of Federal Regulations (CFR), Title 9 (Animals and Animal Products), Subchapter A (Animal Welfare), Parts 1, 2, and 3. Copies of the regulations may be obtained online searching for “Animal Welfare Act” or at awic.nal.usda.gov/government-and-professional-resources/federal-laws/animal-welfare-act. The relevant regulations and standards covered by the AWA are summarized as follows.

Subjects Addressed by the AWA

- Part 1: Definition of Terms
- Part 2: Regulations
 - Subpart A: Licensing
 - Subpart B: Registration
 - Subpart C: Research Facilities
 - Subpart D: Attending Veterinarian and Adequate Veterinary Care
 - Subpart E: Identification of Animals
 - Subpart F: Stolen Animals
 - Subpart G: Records
 - Subpart H: Compliance with Standards and Holding Period
 - Subpart I: Miscellaneous

- Part 3: Standards
 - Subparts A–F: Specifications for the Humane Handling, Care, Treatment and Transportation of Dogs and Cats, Guinea Pigs and Hamsters, Rabbits, Nonhuman Primates, Marine Mammals, and Other Warm-Blooded Mammals

According to the AWA, “each dealer, exhibitor, operator of an auction sale, and intermediate handler must comply in all respects with the regulations set forth in Part 2 and the standards set forth in Part 3 for the humane handling, care, treatment, housing, and transportation of animals.”

PUBLIC HEALTH SERVICE REGULATIONS

In 1973, a new policy applying to all Public Health Service (PHS) awardee institutions was drafted. The policy required that institutions conducting PHS-supported research comply with the AWA and the *Guide for the Care and Use of Laboratory Animals*.² Each institution is also required to provide the National Institutes of Health (NIH) with an assurance which gives a detailed plan for research, training, testing, education, experimentation, or demonstration purposes. In essence, the policy requires that institutions take responsibility for the quality of their animal research programs and the conduct of investigators and animal care personnel. In 1985, the PHS Policy on Humane Care and Use of Laboratory Animals by awardee institutions was updated, and the final version of the policy was made effective January 1, 1986. Subsequently, Congress enacted and later revised the *Health Research Extension Act* of November 20, 1985 (P.L. 99-158), which added several key provisions to the PHS policy. Although the policy is not law, it has the same effect because an institution must comply in order to compete for funding for animal-related research from PHS and other funding sources. Key elements of the PHS policy include

- Negotiation of Animal Welfare Assurances which include commitments by awardee institutions concerning animal care and use, training of staff, and occupational health programs for employees
- Establishment of an Institutional Animal Care and Use Committee (IACUC) with defined responsibilities
- Compulsory IACUC review of proposed research projects or proposed significant changes in ongoing research projects funded by PHS
- Detailed requirements for information required in applications for awards
- Specific record keeping requirements to ensure clear accountability for the quality of the institutional program
- Specific reporting requirements which enable funding agencies and the NIH Office of Laboratory Animal Welfare (OLAW) to oversee the entire system

Additional information concerning the PHS policy may be obtained from the OLAW, National Institutes of Health, RKL 1, Suite 360, MSC 7982, 6705 Rockledge Dr., Bethesda, MD 20892–7982.

Each institution subject to the PHS policy is expected to operate its research program in accordance with the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Research and Training. These principles are listed verbatim in the following.

US Government Principles for the Utilization and Care of Vertebrate Animals Used in Research and Training

1. The transportation, care, and use of animals should be in accordance with the AWA (7 U.S.C. 2131 *et seq.*) and other applicable Federal laws, guidelines, and policies.
2. Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.
3. The animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation, and in vitro biological systems should be considered.
4. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals.
5. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents.
6. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure.
7. The living conditions of animals should be appropriate for their species and contribute to their health and comfort. Normally, the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling, and use of the species being maintained or studied. In any case, veterinary care shall be provided as indicated.
8. Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.

9. Where exceptions are required in relation to the provisions of these principles, the decisions should not rest with the investigators directly concerned but should be made, with due regard to Principle II, by an appropriate review group such as an Institutional Animal Care and Use Committee. Such exceptions should not be made solely for the purposes of teaching or demonstration.

GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS

The *Guide for the Care and Use of Laboratory Animals*² (*Guide*) was first published in 1963 under the title *Guide for Laboratory Animal Facilities and Care*. The *Guide* has been revised several times since 1963 with the most recent version (i.e., the eighth edition) issued in 2011. The *Guide* provides information on common laboratory species housed under a variety of circumstances. Although the *Guide* is not intended to be an exhaustive review of all aspects of animal care and use, it does address a number of relevant issues, including physical construction of animal facilities, husbandry, veterinary care, sanitation, and qualifications and training of laboratory personnel. The most recent version of the *Guide* (National Academy of Science ISBN-0-309-15401-4, Revised 2011) included consideration of alternatives to animal use, an emphasis on the 3 Rs, the use of humane end points, the provision of adequate veterinary care, and the appropriate transportation of animals. Copies of the *Guide* may be obtained from the National Academies Press, 500 Fifth Street, NW, Lockbox 285, Washington, DC 20055; Telephone number: (800) 624-6242 or (202) 334-3313 (in the Washington metropolitan area). Website: www.nap.edu. Various topics covered by the *Guide* are listed as follows.

*Topics Included in the Guide for the Care and Use of Laboratory Animals*²

- Key Concepts
 - Applicability and Goals
 - Intended Audiences and Uses of the *Guide*
 - Ethics and Animal Use
 - The Three Rs
 - Key Terms
 - Policies, Principles, and Procedures
- Animal Care and Use Program
 - Regulations, Policies, and Principles
 - Program Management
 - Personnel Management
 - Investigating and Reporting Animal Welfare Concerns
 - Program Oversight and the Role of the IACUC
 - Disaster Planning and Emergency Preparedness
- Environment, Housing, and Management
 - Terrestrial and Aquatic Animals
 - Environment, Microenvironment, and Macroenvironment

- Temperature, Humidity, Ventilation, Water and Air Quality
- Illumination
- Noise and Vibration
- Housing
- Environmental Enrichment
- Sheltered, Outdoor, or Naturalistic Housing Space
- Behavioral and Social Management, Husbandry, and Population Management
- Veterinary Care
 - Animal Procurement and Transportation
 - Preventive Medicine, Animal Biosecurity, Quarantine and Stabilization
 - Separation by Health Status and Species
 - Surveillance, Diagnosis, Treatment, and Control of Disease
 - Clinical Care and Management
 - Surgery
 - Pain and Distress
 - Anesthesia, Analgesia, and Euthanasia
- Physical Plant
 - General Considerations
 - Functional Areas
 - Construction Guidelines
 - Special Facilities
 - Security and Access Control
 - Appendices
- A. Additional Selected References
- B. US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training
- C. Statement of Task
- D. About the Authors

INSTITUTIONAL PROGRAMS

AAALAC INTERNATIONAL

The Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) is a nonprofit corporation whose primary goal is to promote high-quality care and use of animals through a voluntary accreditation program. Institutions maintaining, using, importing, or breeding laboratory animals for scientific research are eligible to apply for AAALAC accreditation. The accreditation process involves inspection of the animal facilities and program by experts in laboratory animal science, who submit a comprehensive report for consideration by the Council on Accreditation. The Council reviews the report, using the *Guide* as a basis for determining whether full accreditation should be granted. If accreditation is granted, facilities are required to submit annual reports concerning the status of their animal facilities and animal program. Site reinspections are conducted by AAALAC representatives at intervals of 3 years or less to determine whether accreditation should be continued.

The specific standards that have been established by the AAALAC International Board of Trustees for accreditation are listed in the following paragraphs (AAALAC.org).

AAALAC Accreditation Standards

- “The care and management of laboratory animals should be directed by qualified persons.
- All animal care personnel should be suitably qualified by training and experience in the care of laboratory animals.
- Physical facilities and the methods of care and use for animals should permit their maintenance in a state of well-being and comfort.
- The *Guide for the Care and Use of Laboratory Animals (Guide)*, Eighth Edition (National Research Council 2011), shall serve as a basic guide to the establishment of specific standards for accreditation. AAALAC International may establish standards based on prevailing directives, conventions and guidelines of the country in which the accreditable unit is located.
- The accreditable unit shall observe any and all statutes and governmental regulations which bear upon animal care and use including, but not limited to, the prevailing standards of sanitation, health, labor, and safety of the jurisdiction(s) in which it is located.
- The accredited unit shall submit an annual report which describes elements of the animal care and use program as specified by AAALAC International. In addition, the accredited unit shall promptly notify AAALAC International (e.g., through copies of correspondence) of adverse events relating to the animal care and use program. Examples include investigations by the USDA or OLAW, as well as other serious incidents or concerns that negatively impact animal well-being.
- Membership in an association or organization for laboratory animal care and use or in an association of persons dedicated to the care and use of laboratory animals or membership in any association or organization shall not be required as a condition for gaining or maintaining accredited status under the AAALAC International accreditation program.”

Once a facility becomes fully accredited, a certificate of accreditation is issued, and the facility is identified on a list of accredited facilities published in the Association's Activities Report. Full AAALAC International accreditation is accepted as partial assurance by NIH that the animal facility and program are in compliance with PHS policy. Further information on AAALAC accreditation may be obtained from AAALAC International, 5283 Corporate Drive, Suite 203, Frederick, MD 21703-2879. Telephone: (301) 696-9626. Fax: (301) 696-9627.

IACUC

The AWA, PHS policy, and *Guide for the Care and Use of Laboratory Animals* all require the establishment of an Institutional Animal Care and Use Committee (IACUC which

is responsible for monitoring the facility's animal care and use program). The AWA requires that the IACUC be appointed by the chief executive officer of the research facility and consist of a chairperson and at least two additional members as follows:

- A Doctor of Veterinary Medicine, with training or experience in laboratory animal science and medicine, who has direct or delegated program responsibility for activities involving the research facility.
- An individual not affiliated in any way with the facility other than as a member of the committee, and not a member of the immediate family of a person who is affiliated with the facility. This individual should provide representation for general community interests in the proper care and treatment of animals.

The *Guide for the Care and Use of Laboratory Animals*² specifies an IACUC composition comparable to that required by the AWA, with the addition of "other members as required by institutional needs and by federal, state and local regulations and policies." The PHS policy is somewhat different in that it specifically requires that the IACUC be composed of at least five members, including a veterinarian with program responsibilities, a scientist experienced in laboratory animal research, a nonscientist, and an individual who has no other association with the institution besides membership in the IACUC (the specific role and background of the fifth member is not specified).

The AWA, PHS policy, and *Guide for the Care and Use of Laboratory Animals* all specify relatively similar functions for the IACUC, which include:

- Review, at least once every 6 months, the research facility's program for humane care and use of animals.
- Inspect, at least once every 6 months, all the animal facilities, including animal study areas and satellite facilities.
- Prepare and submit reports of IACUC evaluations to the institutional official.
- Review and, if warranted, investigate concerns involving the care and use of animals at the facility resulting from public complaints and from reports of noncompliance received from facility personnel or employees.
- Make recommendations to the institutional official regarding any aspect of the research facility's animal program, facilities, or personnel training.
- Review and approve, require modifications in (to secure approval), or withhold approval of those components of proposed activities related to the care and use of animals.
- Review and approve, require modifications in (to secure approval), or withhold approval of proposed significant changes regarding the care and use of animals in ongoing activities.

The IACUC may also suspend any activity involving animals which is deemed unacceptable. However, it is the intent of the guidelines mentioned earlier to avoid such situations through the implementation of sensible and ethical animal programs, training, and preliminary review of proposed animal activities by the IACUC.

Further information concerning IACUC authority and functions may be found in the AWA, 9 CFR, Subchapter A, Part 2, Subpart C, 2.3.1. In addition, the second edition of the *Institutional Animal Care and Use Committee Guidebook*, prepared by the NIH OLAW, may be obtained online at <http://grants.nih.gov/grants/olaw/GuideBook.pdf>.

PROFESSIONAL AND GOVERNMENTAL ORGANIZATIONS

Information concerning various professional and governmental organizations involved in laboratory animal science, animal welfare, or related activities is provided in the following sections.

AALAS

The American Association for Laboratory Animal Science (AALAS) is concerned with all aspects of laboratory animal care and use, and provides a means for collection and exchange of information on all phases of animal care and management. The association holds annual meetings and publishes two bimonthly journals, *Comparative Medicine*, an international journal of comparative and experimental medicine, as well as the *Journal of the American Association for Laboratory Animal Science* (JAALAS), the official journal of AALAS. The AALAS Certification and Registry Board offers three levels of technical certification, that is, Assistant Laboratory Animal Technician (ALAT), Laboratory Animal Technician (LAT), and Laboratory Animal Technologist (LATG), as well as a managerial certification, Certified Manager Animal Resources (CMAR). Additional information may be obtained from AALAS. Telephone: (901) 745-8620. Fax: (901) 753-0046. Website: www.aalas.org.

ACLAM

The American Board of Laboratory Medicine was founded in 1957 to encourage education, training, and research; to establish standards of training and experience for qualification; and to certify, by examination, qualified laboratory animal specialists as diplomates. In 1961, the name of the organization was changed to The American College of Laboratory Animal Medicine (ACLAM). The college emphasizes and sponsors continuing education and auto-tutorial programs on the use, husbandry, and diseases of animals used in research. Through its Career Pathways and Mentoring Committees, ACLAM seeks to increase the number of laboratory animal veterinarians in biomedical research. Additional information may be obtained through the ACLAM website at www.aclam.org/.

ASLAP

The American Society of Laboratory Animal Practitioners (ASLAP) was organized to disseminate ideas, experience, and knowledge among veterinarians involved in laboratory animal practice through education, training, and research. The Society was founded in 1966, and membership is open to all veterinarians, veterinary residents, and veterinary students. ASLAP holds two educational meetings annually, one in conjunction with the AVMA annual meeting and one in conjunction with the AALAS annual meeting. Additional information may be obtained through the ASLAP Coordinator, 9190 Crestwyn Hills Drive, Memphis, TN 38125-8538. Telephone: (901) 333-0498. Fax: (901) 753-0046. Website: www.aslap.org/.

AVMA

The American Veterinary Medical Association (AVMA), established in 1863, is the major US national organization of veterinarians and is the largest veterinary medical association in the world. The primary mission of the AVMA is the advancement of veterinary medical science, including its relationship to public health, biological science, and agriculture. The AVMA Council of Education is the major accrediting agency for US colleges of veterinary medicine, but has also accredited schools of veterinary medicine in other countries including Australia, Canada, Ireland, Mexico, and the United Kingdom. The AVMA Committee on Veterinary Technician Education and Training accredits 2-year and 4-year programs in veterinary technology throughout the United States. The AVMA publishes two journals, the *Journal of the American Veterinary Medical Association (JAVMA)* and the *American Journal of Veterinary Research*. The AVMA sponsors specialization in veterinary medicine through the recognition of specialty certifying organizations such as ACLAM. The AVMA also provides state and federal legislators, the media, and the public with information on animal welfare, agricultural practices and products, public health issues, the role of veterinarians in homeland security, and veterinary pharmaceuticals, biologics, and technologies. Additional information may be obtained from the AVMA, 1931 North Meacham Road, Suite 100, Schaumburg, IL 60173. Telephone: (800) 248-2862. Fax: (847) 925-1329. Website: www.avma.org/.

CCAC

The Canadian Council on Animal Care (CCAC) was created in 1968 to oversee, set, and maintain standards for the ethical use of animals in science (research, teaching, testing, and production) in Canada. Through its Guidelines Program, the CCAC issues guideline documents and policy statements on animal use in science that apply throughout Canada. CCAC's Assessment and Certification Program approves animal care and use programs of any organization that uses animals in science-related research, teaching, testing, or production. CCAC develops educational and training materials for all personnel involved with the ethical use

of animals in science through its Education, Training, and Communications Program. In addition, the CCAC's Three Rs Program promotes and supports projects that implement the three "R"s, that is, replacement, reduction, and refinement. CCAC also issues an annual report to the general public that includes comprehensive annual statistics on the number of animals used in science. For further information, contact CCAC at 130 Albert St., Suite 1510, Ottawa, ON, Canada, K1P 5G4. Telephone: (613) 238-4031. Fax: (613) 238-2837. Website: www.ccac.org/.

ECLAM

The European College of Laboratory Animal Medicine (ECLAM) was founded in 2000 and was awarded full recognition in 2008 by the European Board of Veterinary Specialization to oversee and promote the practice of laboratory animal medicine in Europe. ECLAM seeks to improve: the prevention, diagnosis, and treatment of laboratory animal diseases; the prevention and alleviation of pain and distress; the training of individuals involved in research using laboratory animals; the design of laboratory animal facilities; control of the animals' environment; the selection and production of laboratory animals; and the design and conduct of research involving laboratory animals. Like ACLAM, ECLAM certifies qualified laboratory animal specialists as diplomates in laboratory animal medicine. Additional information may be obtained through the ECLAM website at www.eclam.org/.

ICLAS

The International Council for Laboratory Animal Science (ICLAS) is a nongovernmental organization that encourages international cooperation in laboratory animal science. ICLAS promotes the development of international standards for the care and use of laboratory animals, disseminates information concerning laboratory animals, sponsors scholarships for education, and supports programs that advance laboratory animal science in developing nations. ICLAS issues the *ICLAS Bulletin* every spring and autumn. Additional information may be obtained from www.iclas.org.

ILAR

The Institute of Laboratory Animal Resources (ILAR) was founded in 1952, and is a unit in the Division on Earth and Life Studies of the National Research Council. ILAR's mission is to provide expert counsel to the federal government, the international biomedical research community, and the public on the scientific, technological, and ethical use of laboratory animals through the issuance of expert committee reports, the *ILAR Journal*, web-based resources, and other means. ILAR promotes the high-quality humane care of laboratory animals; the appropriate use of laboratory animals; and the exploration of alternatives in research, testing, and teaching. The most recent edition of the *Guide for the Care and Use of Laboratory Animals*² was prepared by ILAR for

the NIH. For more information, contact ILAR, 500 Fifth Street NW, Washington, DC 20001. Telephone: (202) 334-2590. Fax: (202) 334-1687. E-mail: fsharples@nas.edu.

SCAW

The Scientists Center for Animal Welfare (SCAW) was founded in 1979 and consists of individuals and institutions concerned with various aspects of animal welfare. SCAW promotes the principle of humane animal care and treatment in all areas of animal science. Among other activities, SCAW develops educational materials and national guidelines on humane animal experimentation, monitors animal legislation issues, sponsors an educational winter conference, and presents or conducts seminars, workshops, and surveys. For more information, contact SCAW, 7833 Walker Drive, Suite 410, Greenbelt, MD 20770. Telephone: (301) 345-3500. Fax: (301) 345-3503. E-mail: info@scaw.com. Website: www.scaw.com.

NIH

The National Institutes of Health (NIH) is the principal US federal agency that disburses funds for biomedical research. The NIH also sets PHS policy on laboratory animal welfare. Additional information may be obtained from the Office of Animal Care and Use, 9000 Rockville Pike, Bethesda, MD 20892. Telephone: (301) 496-5424.

DEA

The Drug Enforcement Administration (DEA) of the US Department of Justice is the regulatory authority responsible for the enforcement of laws pertaining to controlled substances. Licenses to use controlled substances are obtained from this agency. Additional information may be obtained from the US Department of Justice, Drug Enforcement Administration, Attn: Registration Section ODR, PO Box 2639, Springfield, VA 22152-2639.

FDA

The US Food and Drug Administration (FDA) protects public health through the oversight of food safety, dietary supplements, pharmaceuticals and biopharmaceuticals, vaccines, veterinary products, medical devices, cosmetics, radiation emitting devices, and tobacco products. The FDA created and enforces the Good Laboratory Practice (GLP) regulations for nonclinical laboratory studies conducted to support applications for research or marketing permits for products regulated by this agency. Additional information may be obtained from the US Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD 20993. Telephone: (888) 463-6332. Website: www.fda.gov/.

EPA

The mission of the US Environmental Protection Agency (EPA) is to protect both human health and the environment. The EPA

has promulgated Good Laboratory Practice regulations under both the Toxic Substances Control Act and the Federal Insecticide, Fungicide, and Rodenticide Act. In addition, the EPA's Office of Chemical Safety and Pollution Prevention has issued harmonized guidelines for use in the testing of toxic chemicals and pesticides in animal models. Additional information may be obtained at the EPA website at www.epa.gov/. Telephone numbers and other contact information for the EPA's 10 regional offices can be found at the website.

OLAW

The Officer of Laboratory Animal Welfare (OLAW) of the NIH oversees the PHS Policy on Humane Care and Use of Laboratory Animals by providing interpretation and guidance on the PHS Policy, by supporting educational programs, and by monitoring the compliance of both assured institutions and PHS funding components. Additional information may be obtained from: Office of Laboratory Animal Welfare, NIH, RKL 1, Suite 360, MSC 7982, 6705 Rockledge Dr., Bethesda, MD 20892-7982 (US Mail) or 20817 (overnight courier). Telephone: (301) 496-7163.

APHIS

The Animal, Plant, and Health Inspection Service (APHIS) is the division of the US Department of Agriculture that administers the federal AWA. To administer and enforce the provisions of the AWA, APHIS issues licenses and registrations to businesses and research facilities that use animals and perform regular inspections to ensure the appropriate care and humane treatment of animals. Additional information may be obtained from USDA, APHIS, 2568-A Riva Road, Annapolis, MD 21401. Telephone: (410) 571-8692. Website: www.aphis.usda.gov.

AWIC

The Animal Welfare Information Center (AWIC) is an information center of the USDA National Agricultural Library established in 1986 as the result of the 1985 amendment to the AWA. Additional information may be obtained from Animal Welfare Information Center, National Agricultural Library, 10301 Baltimore Avenue, Room 410, Beltsville, MD 20705. Telephone: (301) 504-6212.

CAAT

The Center for Alternatives to Animal Testing (CAAT), established in 1981, is a part of the Johns Hopkins University Bloomberg School of Public Health and supports the creation, development, validation, and use of alternatives to animals in research, product safety testing, and education. The center supports grants, sponsors symposia, and publishes a variety of materials related to animal testing and alternatives. Additional information may be obtained from The Johns Hopkins Center for Alternatives to Animal Testing (CAAT), Bloomberg School of Public Health, Department of

Environmental Health Sciences, 615 N. Wolfe St., W7032, Baltimore, MD 21205. Telephone: (410) 614-4990. Fax: (410) 614-2871. E-mail: caat@jhsph.edu.

NABR

The National Association for Biomedical Research (NABR) is a nonprofit organization which was established in 1979 and merged with the National Society for Medical Research in 1985. NABR members include universities, medical and veterinary schools, health agencies, academic and professional societies, and private and public research organizations. NABR actively lobbies Congress, the USDA, NIH, FDA, CDC, and the White House on legislative and regulatory matters affecting laboratory animal research on behalf of its members by encouraging legislation, by commenting on legislative proposals, by testifying at Congressional hearings, and by serving as a resource for Congressional staff and constituents. NABR played a critical role in securing the passage of the Animal Enterprise Terrorism Act in 2006.

NABR supports the responsible and ethical use of laboratory animals in research, education, and product safety testing. NABR recognizes that it may not be feasible to completely replace live animals in research because whole living organisms are an indispensable element of biomedical research and testing. Still, the Association believes that animal use should be minimized whenever possible; that pain and distress should be avoided and/or minimized; and that alternatives to live animals should continue to be developed and utilized, whenever feasible. Additional information may be obtained from National Association for Biomedical Research, 818 Connecticut Ave., NW, Suite 900, Washington, DC 20006. Telephone: (202) 857-0540. Fax: (202) 659-1902. E-mail: info@nabr.org. Website: www.nabr.org.

OECD

The Organization for Economic Cooperation and Development (OECD) was established in 1961. Its mission is to promote policies to improve the economic and social well-being of its member nations. With regard to chemical safety testing, OECD has generated specific testing guidelines, guidance documents for humane end points and the use of alternative models, and its own Good Laboratory Practice regulations. Additional information may be obtained at the OECD website at www.oecd.org/.

ORGANIZATIONS THAT OPPOSE THE USE OF ANIMALS IN RESEARCH

A number of organizations strongly oppose the use of animals in research, or express different philosophies regarding this subject. There are, however, some deep divisions in the philosophies and strategies of these organizations. For example, animal welfare groups such as the humane societies tend to be most concerned with the proper care and treatment

of animals, pet adoption, and humane euthanasia. On the other hand, "animal rights" groups are primarily concerned with establishing the "legal rights" of animals. These latter groups outwardly oppose the use of animals in research and the "exploitation" of animals for sport or food. As a result of an increasing number of incidents in which tactics of violence and intimidation were used against members of the biomedical research community by animal rights activists, the Animal Enterprise Terrorism Act (AETA) was enacted into law in the United States in November of 2006. This act expands criminal prohibitions against the use of force, violence, and intimidation involving animal enterprises, and increases the penalties for any violations of such prohibitions.

The activities of such groups have challenged the research community to better inform and educate the public about the critical need for animals in research. Additional standing goals for the biomedical research community include

- Reduction of the number of animals used through thoughtful selection of techniques, models, and experimental designs
- Relief of any unavoidable discomfort to animals
- Improvement of animal facilities and assurance that personnel are fully informed and properly trained
- Elimination or reduction of experimental procedures that cause pain or distress
- Use of nonanimal alternatives where possible

ANIMAL PAIN, THE 3 Rs, AND HUMANE END POINTS

ANIMAL PAIN

In accordance with the AWA, the *Guide for the Care and Use of Laboratory Animals*, and Public Health Service Policy for the Humane Care and Use of Laboratory Animals, veterinarians and investigators must identify and eliminate sources of pain and distress, with the exception of those procedures that are essential to the research in question and approved by the IACUC. Although it is widely agreed that laboratory animals need not experience substantial pain or distress, there is a general lack of agreement on the specific meaning of such terms as comfort, well-being, discomfort, stress, fear, anxiety, pain, and distress. Nonetheless, provisional definitions for these terms have been developed³ and are presented as follows:

- *Comfort*: A state of physiological, psychological, and behavioral equilibrium in which an animal is accustomed to its environment and engages in normal activities, such as feeding, drinking, grooming, social interaction, sleeping-waking cycles, and reproduction.
- *Well-being*: A positive mental state that reflects the level of welfare and comfort of an animal.
- *Discomfort*: A minimal change in an animal's adaptive level or baseline state as a result of

changes in its environment or biological, physical, social, or psychotic alterations. Physiological or behavioral changes that indicate a state of stress might be observed, but are not marked enough to indicate distress.

- *Stress*: The effect produced by external (physical or environmental) events or internal (physiological or psychological) factors, referred to as stressors, which induce an alteration in an animal's biological equilibrium.
- *Anxiety and fear*: Emotional states that are traditionally associated with stress. They can be adaptive in that they inhibit an organism's actions that could lead to harm or cause it to act in ways allowing it to escape from potentially harmful situations.
- *Pain*: Results from potential or actual tissue damage. Pain can be considered a potent source of stress, that is, a stressor. It can also be considered a state of stress itself, however, and can lead to distress and maladaptive behaviors.
- *Distress*: An adverse state in which an animal is unable to adapt completely to stressors and the resulting stress and shows maladaptive behaviors. It can be evident in the presence of various experimental or environmental phenomena, such as abnormal feeding, absence or diminution of postprandial grooming, inappropriate social interaction with conspecifics or handlers, and inefficient reproduction.

With regard to the issues of animal discomfort and pain, researchers should consider the following questions before undertaking any live animal experiment:

- Will the procedure yield results that are beneficial to animal or human health and well-being?
- Has a literature search been performed to ensure that the proposed procedures do not unnecessarily duplicate previous experiments?
- Is the species and number of animals appropriate for the purpose of the experiment?
- Is the discomfort to the animals limited to that which is unavoidable in the conduct of the experiment?
- Have appropriate analgesic, anesthetic, and tranquilizing drugs been considered to minimize pain and discomfort?
- Has the method of euthanasia been considered?
- Are the individuals performing the experimental procedures and caring for the animals properly trained?

THE 3 Rs

Introduced by Russell and Burch⁴ in 1959, the 3 Rs stand for replacement, reduction, and refinement. *Replacement* refers to testing methods or paradigms that either avoid or entirely replace the use of nonhuman vertebrates.

Examples include the use of established cell lines, tissue engineering, mathematical and computer modeling, and the use of invertebrate testing systems. *Reduction* refers to testing methods or paradigms that minimize the actual number of animals used. Examples of reduction include: improvements in experimental design such as the incorporation of safety pharmacology parameters in standard toxicology studies to avoid conducting stand-alone safety pharmacology studies, the elimination of poorly controlled variables, or reduction in the number of control animals used where feasible; the use of transgenic mouse alternatives (~25/sex/group) to the two-year mouse carcinogenicity study (≥50/sex/group); and the sharing of data by two or more institutions to avoid unnecessary or duplicative experimentation. *Refinement* refers to changes in experimental procedures or husbandry to minimize pain and distress or result in improvement in animal welfare in those situations in which animals must be used. Refinement can actually improve data quality through the minimization of intra-animal and inter-animal variability. Examples include the use of noninvasive techniques for data collection, the provision of adequate anesthesia or analgesia when pain may be induced, the provision of environmental enrichment to improved living conditions for laboratory animals, and the use of humane end points to allow euthanasia prior to moribundity.

HUMANE END POINTS

The use of humane end points qualifies as refinement under the 3 Rs paradigm by providing alternatives to those experimental end points that result in severe animal pain and distress. A humane end point may be defined as the earliest indicator of severe pain, severe distress, suffering, or impending death in an animal experiment. The purpose of using humane end points in animal experiments is to predict severe pain, severe distress, suffering, or impending death, before the animal experiences these effects. Some general categories useful for monitoring pain and distress include: changes in physical appearance, changes in clinical signs, changes in unprovoked behavior, behavioral changes in response to external stimuli, changes in clinical parameters, and changes in body weight and food/water consumption. Signs of moribundity or impending death include, but are not limited to: prolonged, impaired ambulation, prolonged anorexia, excessive weight loss, severe dehydration, significant blood loss, evidence of organ failure, prolonged absence of voluntary responses to external stimuli, persistent, labored breathing, the continued inability to remain upright, persistent convulsions, self-mutilation, prolonged diarrhea, significant and sustained decreases in body temperature, and tumors of a substantial size.

In Table 1.1, the findings of moderate severity would ideally be used as humane end points, and the findings of substantial severity would clearly warrant the humane euthanasia of study animals.

TABLE 1.1
End Point Guidance for Rodents

Finding	Mild Severity	Moderate Severity	Substantial Severity
Weight	Reduced weight gain	Weight loss $\leq 20\%$	Weight loss $> 25\%$
Food/water consumption	40%–75% of normal for 72 h	$< 40\%$ of normal for 72 h	$< 40\%$ of normal for 7 days, or anorexic for 72 h
Piloerection	Partial	Marked	Marked with other signs of dehydration
Response to provocation	Subdued with normal response	Subdued response	Unresponsive
Interaction with peers	Interaction	Little interaction	No interaction
Hunched posture	Transient	Intermittent	Persistent
Vocalization	Transient	Intermittent when provoked	Unprovoked
Oculonasal discharge (chromorhinodacryorrhea)	Transient	Persistent	Persistent and copious
Respiration	Normal	Intermittent abnormal	Labored
Tremors	Transient	Intermittent	Persistent
Convulsions	Absent	Transient	Prolonged
Prostration	Absent	Transient (< 1 h)	Prolonged (> 1 h)
Self-mutilation	Absent	Absent	Present

Source: Adapted from Robinson, S. et al., *Guidance on Dose Level Selection for Regulatory General Toxicology Studies for Pharmaceuticals*, Laboratory Animal Science Association and National Centre for the Replacement, Refinement, and Reduction of Animals in Research, December, 2009.

ANIMAL FACILITY SAFETY

The Occupational Safety and Health Act (OSHA), which is administered by the US Department of Labor, is not specifically directed at laboratories and research operations. However, the regulations apply to all workplaces and cover fire, electrical, and mechanical safety, and exposure to chemicals, radiation, and noise. In general, research laboratories have not been subjected to the frequent and rigorous OSHA inspections which are common to industries with intrinsically high accident rates. However, there now exist specific OSHA regulations concerning occupational exposures to toxic substances in research laboratories. These standards require that the laboratory develop a “Chemical Hygiene Program” designed to provide employee protection in the specific circumstances of the individual laboratory. There are also requirements for training of employees, worker availability to reference materials concerning chemical hazards, and a provision for medical consultation and examination. In addition, both AAALAC and the *Guide for the Care and Use of Laboratory Animals* strongly emphasize occupational safety and health in laboratory animal facilities.

ZOONOTIC DISEASES^{5,11}

Zoonotic diseases are those which are transmissible from animals to humans under natural conditions. A few of the better known zoonotic diseases are described in the following sections.

HEPATITIS

Hepatitis A virus can infect chimpanzees, gorillas, patas monkeys, celebres, apes, woolly monkeys, and some tamarins. However, chimps recently introduced into captivity are the most common source of infection for humans. The incubation period for the virus may be 15–50 days, followed by abrupt onset of fever, anorexia, nausea, and jaundice. The severity of the disease is related to age, with fatality quite low among hospitalized patients. Lifelong immunity is conferred by development of an IgG immune response. Disease control measures involve quarantine, adequate protective clothing, sanitation, and personal hygiene. As an additional measure, the PHS recommends immunoprophylaxis (i.e., administration of immune serum globulin every 4 months) for personnel in close contact with newly imported chimps.

HERPESVIRUS B

Herpesvirus B, which is caused by *Herpesvirus simiae*, represents the most serious health hazard to humans from non-human primates. In the natural host of the virus, the *Macaca* spp, the disease is mild and similar to that of herpes simplex in humans, with the development of tongue and lip ulcers which heal in 7–14 days. In contrast, in infected humans, disease symptoms may be similar to polio, with rapid flaccid paralysis leading to death, or permanent paralysis in survivors. Transmission usually occurs through a bite from an

infected animal, or by exposure of the broken skin or mucus membranes to infected saliva or infected tissues. Because of the potential danger to humans, all macaques should be viewed as potential carriers, and protective clothing should be worn at all times which protects the handler from bites and scratches. Although antiserum is available, its effectiveness is questionable.

Note: Herpes simplex in man can be transmitted to lower primates with generalized disease in owl monkeys, tree shrews, lemurs, marmosets, and tamaris.

RABIES

In the United States, the skunk and bat are the largest natural reservoirs of the rabies virus. The virus is transmitted through the saliva of infected animals via bites, scratches, abrasions, or across mucus membranes. In dogs, the virus is present in the saliva for 1–14 days before clinical symptoms manifest. In humans, the disease is almost always fatal, even when proper treatment is begun shortly after exposure. The most important disease control measure for domestic animals is vaccination. For humans, preexposure vaccination should be made available for all persons working with potentially infected animals.

LYMPHOCYTIC CHORIOMENINGITIS

Mice, hamsters, and humans serve as natural hosts for the virus causing lymphocytic choriomeningitis, and wild mice are a natural reservoir for the virus, which is the

only latent virus in mice that naturally infects humans. The incidence of the disease may be 100% in wild populations, and may become 100% in breeding colonies if preventive measures are not instituted. However, only persistently infected mice and acutely infected hamsters are known to transmit the virus, which may be passed in the urine, feces, saliva, and nasal secretions of carrier animals. Lifelong infection with high concentrations in all organs is often observed in fetal and newborn infected mice. Hamsters, on the other hand, may remain infected for long periods, but eventually eliminate the virus. There are four different recognized forms of the disease in mice. In the cerebral form, death may occur with no previous symptoms on the fifth or sixth day after inoculation. In the visceral form, death may also occur after several days, but is often preceded by conjunctivitis and ruffled fur. In the late-onset form which occurs in neonatally infected mice, animals may seem healthy until 9–12 months of age when signs of chronic illness manifest, including ruffled fur, weight loss, and hunched posture. A form resulting in early death of neonatally exposed mice may also occur under poorly understood conditions. Infection of humans often results in mild influenza-like symptoms, and may or may not involve the central nervous system (CNS). Other than direct virus isolation, a rise in antibody titer serves as the most conclusive diagnosis of infection.

OTHER ZOONOSES

Other zoonoses are described briefly in Table 1.2.

TABLE 1.2
Other Zoonotic Diseases

Disease	Description
Monkey pox	Related to smallpox; clinical signs in humans include fever, headache, sore throat, and rash
Benign epidermal monkey pox (BEMP)	Primarily affects macaques and Leaf monkeys; circumscribed elevated lesions on eyelids, face, and elsewhere; in humans, disease regresses in 2–3 weeks
Yaba virus	Caused by a poxvirus transmitted via a mosquito vector; virus has been inoculated into humans, but natural transmission has not been recorded; infected animals develop benign histiocytomas that eventually regress
Contagious ecthyma (ORF)	Caused by poxvirus of sheep and goats; characterized by epithelial proliferation and necrosis in the skin and mucous membranes of urogenital and gastrointestinal tracts; in humans, seen as painful nodules on hands which resolve in 1–2 months
Yellow fever	Caused by an RNA flavivirus, transmitted by mosquitos; classic lesion is massive hepatic midzonal necrosis; disease severity varies among species of nonhuman primates
Hantaviral diseases (Korean hemorrhagic fever, epidemic hemorrhagic fever, Hantavirus pulmonary syndrome, nephropathia epidemica)	Caused by <i>Hantaan</i> virus carried by wild rodents worldwide; disease involves fever and renal and/or pulmonary involvement with headaches, diarrhea, nausea, vomiting, and possible hemorrhagic symptoms
Measles	Caused by a morbillivirus of the family <i>Paramyxoviridae</i> . Highly contagious disease with incubation time of 9–11 days; rash begins in oral cavity and spreads over face, neck, chest, and body; natural immunity develops after capture, but vaccination may be necessary for naive animals. Measles is not a natural disease of macaques, but is acquired through contact with humans
Rickettsialpox	Caused by <i>Rickettsia akarii</i> ; domestic mice are natural host, and vector is the mite, <i>Allodermanyssus sanguineus</i> ; self-limiting disease in man characterized by fever, headache, myalgia, lymphadenopathy, leukopenia, eschar-like lesions, and generalized rash
Murine typhus	Caused by <i>Rickettsia typhi</i> ; transmission to humans via rat fleas; clinical signs similar to those of rickettsialpox

(continued)

TABLE 1.2 (continued)
Other Zoonotic Diseases

Disease	Description
Rocky Mountain spotted fever	Caused by <i>Rickettsia rickettsii</i> ; transmitted by ticks (<i>Dermacentor</i> spp.) as vectors and reservoir hosts; mammalian hosts include wild rodents, lagamorphs, and dogs; disease in humans includes fever, headache, myalgia, and generalized hemorrhagic rash
Q fever	Caused by <i>Coxiella burnetii</i> ; disease is widespread in sheep; dogs, cats, and chicken can become infected; organism is shed in urine, feces, milk, and placenta of asymptomatic ungulates; incubation is 2–3 weeks and results in febrile systemic disease; most cases resolve in 2 weeks
Psittacosis	Caused by <i>Chlamydia psittaci</i> ; hosts include mice, guinea pigs, rabbits, cats, lambs, calves, birds, and frogs; disease includes conjunctivitis, pneumonitis, pericarditis, hepatitis, enteritis, urethritis, and arthritis; in humans, may be asymptomatic or present after 1–2 weeks of incubation, frequently with respiratory symptoms
Brucellosis	Caused in the laboratory by <i>Brucella canis</i> , due to use of random-source dogs; oral and transcutaneous routes of infection occur in the laboratory; brucellosis should be suspected when dog has history of abortion or infertility; source of infection is not known in most human cases. Other <i>Brucella</i> species may be contracted through the use of other species in the laboratory environment, that is, goats, sheep, pigs, cattle
Leptospirosis	<i>Leptospira</i> spp. bacteria are found worldwide and divided into serovars based on DNA-relatedness; reservoirs are wild and domestic animals, including rats, swine, cattle, and dogs; transmission primarily via contact with skin, especially if abraded, or mucous membranes with infected urine-contaminated materials; may be clinically inapparent or present with fever of sudden onset, headache, chills, severe myalgia, conjunctival suffusion, or may present with a diphasic fever, meningitis, rash, hemolytic anemia, hemorrhage into skin and mucous membranes, hepatorenal failure, jaundice, mental confusion/depression, myocarditis, and pulmonary involvement
Tuberculosis	Caused by <i>Mycobacterium</i> acid-fast bacilli; natural reservoirs are cattle, birds, and humans, with many other species susceptible; outbreaks occur in nonhuman primates, with Old World species more susceptible than New World monkeys and great apes; tuberculosis can occur in every organ system, although respiratory system is the most familiar form
Campylobacteriosis	Caused by <i>Campylobacter</i> spp. which has been isolated from dogs, cats, hamsters, ferrets, nonhuman primates, rabbits, swine, cattle, sheep, chicken, turkeys, and wild birds; disease in humans is self-limiting and usually brief; clinical symptoms include abdominal pain, fever, and diarrhea
Salmonella	Caused by over 1600 serotypes worldwide; two most common in laboratory colonies are <i>Salmonella typhimurium</i> and <i>Salmonella enteritidis</i> , due primarily to contaminated laboratory feed; acute gastroenteritis is the most common presenting symptom; some cases proceed to septicemia after bacterial invasion of gut wall
Shigellosis	Caused by <i>Shigella</i> spp., including <i>S. flexneri</i> , <i>S. sonnei</i> , and <i>S. dysenteriae</i> , all found in nonhuman primates; humans are the main reservoirs; nonhuman primates acquire disease after contact with infected primates or through contaminated facilities, food, or water; children may exhibit more severe disease with symptoms of dysentery with blood and mucus in feces
<i>Streptobacillus moniliformis</i>	Common in wild rodents, rare in laboratory rats, causes rat-bite fever (Haverhill Fever) in man; organism inhabits oropharynx of rat, and is transmitted by bite
<i>Yersinia</i>	Species which are zoonotic in laboratory animals include <i>Y. pseudotuberculosis</i> , <i>Y. enterocolitica</i> , and <i>Y. pestis</i> ; <i>Y. pseudotuberculosis</i> and <i>Y. enterocolitica</i> produce mesenteric lymphadenitis, septicemia, and appendicitis in humans; infection can occur through feces-contaminated food, or through direct contact with infected animals
Dermatophilosis	Caused by <i>Dermatophilus congolensis</i> ; experimentally transmitted to mice, guinea pigs, and rabbits; produces circumscribed patches of alopecia in infected animals with exudative dermatitis; organism may persist in the fur and infect humans
Erysipeloid	Caused by <i>Erysipelothrix rhusiopathiae</i> in swine, lambs, calves, poultry, fish, and wild and laboratory mice; produces inflammatory lesions of the skin with occasional concurrent septicemia; pigs are the most common source in the laboratory
Listeriosis	Caused by <i>Listeria monocytogenes</i> ; laboratory species most commonly affected are ruminants, guinea pigs, rabbits, and chinchillas; in normal hosts, disease may be expressed as pustular or papular cutaneous lesions or an acute, mild, febrile illness, sometimes with influenza-like symptoms; pregnant women and fetuses are at risk with the potential for <i>in utero</i> infections and abortion
Pseudomonas	Opportunistic organism, especially for immunosuppressed animals; transmission from the caretakers or animals has been documented, but not the reverse
Dermatomycoses (ringworm)	Caused by three genera of fungi: <i>Microsporum</i> , <i>Trichophyton</i> , and <i>Epidermophyton</i> ; frequently, the animals are asymptomatic and not identified until caretaker develops the disease; transmission occurs by direct or indirect contact with infected animal; dermatomycosis is usually self-limiting in humans, and presents as scaling, erythema, and occasional vesicles in the skin

TABLE 1.2 (continued)
Other Zoonotic Diseases

Disease	Description
Toxoplasmosis	Caused by <i>Toxoplasma gondii</i> ; felines develop intestinal infection followed by shedding of oocysts resulting in transmission to humans; human infection is common, but clinical symptoms rare; congenital infection can lead to systemic disease with neuropathological lesions
Amebiasis	Caused by <i>Entamoeba histolytica</i> ; parasite is commonly found in feces of normal monkeys and apes, but may also cause severe clinical disease; most cases of human disease exhibit no clinical symptoms; mild diarrhea to acute bloody or mucoid dysentery with fever or chills may occur after invasion of colon wall
Balantidiasis	Caused by <i>Balantidium coli</i> ; common in domestic swine and also found in humans, great apes, and several monkey species; most infections are asymptomatic
Giardiasis	Caused by <i>Giardia</i> spp.; found worldwide among all classes of vertebrates with no apparent host specificity; dogs and nonhuman primates may serve as reservoirs for human infection; in humans, infection often causes chronic or intermittent diarrhea, with light-colored, soft, and mucoid stools
Pneumocystis pneumonia	Caused by <i>Pneumocystis carinii</i> ; latent infections occur in rodents, lagomorphs, nonhuman primates, and domestic and zoo animals; zoonotic transmission has not been proven but may be possible; disease occurs in immunodeficient individuals or those with other debilities; frequently fatal and characterized by alveolitis as lungs fill with white foamy fluid-containing parasites
Cryptosporidiosis	Caused by <i>Cryptosporidium parvum</i> ; affects epithelial cells of GI, biliary, and respiratory tract of humans, birds, fish, reptiles, rodents, cats, dogs, cattle, and sheep; diarrhea is the major symptom in man, remitting in <30 days in most immunologically competent people; may be fatal in immunodeficient persons

RECOGNITION AND CONTROL OF DISEASE

Adequate veterinary care and daily observation of animals are essential for the recognition and control of disease. Diseases are transmitted by the following routes:

- **Vector:** A living carrier that transfers an infective agent from one host to another.
- **Fomite:** An inanimate object that is not intrinsically harmful, but is able to harbor pathogenic microorganisms.
- **Genes:** Inheritable abnormalities and mutations may result in disease states.

There are several procedures that can be instituted to control disease. Some routine procedures are listed as follows:

- Closely observe each animal at the time of receipt, and reject any animal(s) exhibiting abnormal physical, behavioral, or physiological conditions.

- Isolate and quarantine each new shipment of animals until their health status can be verified.
- Establish procedures that maintain barriers between animals and personnel (e.g., gloves, masks, and protective clothing); between animals and animals (e.g., changing gloves and disinfecting equipment between animals); and between animals and equipment (e.g., disinfect cleaning utensils and sanitize caging).
- Establish animal health and monitoring programs matched to the quality and types of animals and needs of the research laboratory.

Daily observation of animals allows early detection of signs of disease. While checking the general physical condition of each animal, the caretaker should also look for any signs of injury and/or abnormal physiological findings. Observations of any of the conditions listed in Table 1.3 should be followed by diagnosis, prognosis, and treatment, after consultation with the attending veterinarian. If necessary, animals should be euthanized to control disease and alleviate pain and distress.

TABLE 1.3
Abnormal Conditions in Laboratory Animals

Abnormal physical conditions	Dehydrated, emaciated, listless, prostrate, dyspnea, alopecia, circling/head tilt, coughing, sneezing, discharges, scratching, unkempt, abscess/tumor(s), diarrhea, few or no feces, blood in feces, worms in feces, vomitus, bloody vomitus, worms in vomitus
Nonspecific signs of injury	Limping, paralysis, ataxia, dilated pupils, convulsions, fractures, hemorrhage, wounds, contusions
Abnormal physiological findings	Lack of urine, excess urination, few or no feces, anorexia, decreased water intake, excessive water intake

ANIMAL NUTRITION

All animals require regular amounts of clean pure water and food. Fortunately, there are a variety of “complete balanced diets” available commercially for various laboratory species. These diets have been designed to provide the necessary fats, carbohydrates, proteins, fiber, vitamins, and minerals needed by the particular species. Researchers often select “certified” diets for use in their laboratories because these have been assayed for levels of various potential contaminants (e.g., aflatoxins and heavy metals; chlorinated hydrocarbons and

polychlorinated biphenyls; and organophosphate pesticides). Similarly, in many laboratories, the water supplied to the animals is analyzed at regular intervals to ensure potability and absence of contaminants which may negatively impact animal health and research objectives. It is advisable that researchers closely review and retain all reports of food and water analyses. Some of the various types and sources of commercial laboratory diets are listed in Table 1.4. Various nutritional deficiencies which may affect laboratory animals are presented in Table 1.5.

TABLE 1.4
Types and Sources of Commercial Laboratory Diets

Source	Species	Diet Types
Purina Mills, Inc., 505 N., 4th St., Richmond, IN 47374 (765) 962-9561 www.labdiet.com	Rat/mouse/ hamster	5001 Laboratory Rodent Diet; 5002 Certified Rodent Diet; 5008 Formulab Diet; 5010 Laboratory Autoclavable Rodent Diet; 5014 Certified Autoclavable Rodent Diet; 5L36 Certified Rodent Opti-Diet; 5P07 Prolab RMH 1000; 5P06 Prolab RMH 2000; 5P14 Prolab RMH 2500; 5R24 Autoclavable Prolab RMH 2500; 5P00 Prolab RMH 3000; 5P04 Autoclavable Prolab RMH 3500; 5053 Pico [®] Lab Rodent Diet 20; 5061 Pico-Vac Lab Rodent Diet; 5P75 and 5P76 Prolab Isopro RMH 3000
	Rat	5012 Rat Diet
	Mouse	5015 Mouse Diet; 5020 Mouse Diet 9F; 5021 Autoclavable Mouse Breeder Diet; 5058 PicoLab Rodent Diet 20; 5062 Pico-Vac Mouse Diet 20
	Rabbit	5304 Autoclavable Rabbit Diet; 5321 Laboratory Rabbit Diet; 5322 Certified Rabbit Diet; 5325 Certified High Fiber Rabbit Diet; 5326 Laboratory Rabbit Diet HF; 5P25 Prolab Hi-Fiber Rabbit; 5p26 Prolab Rabbit Diet
	Guinea Pig	5025 Guinea Pig Diet; 5026 Certified Guinea Pig Diet; 5L08 Guinea Pig Diet, Autoclavable 20; 5P18 Prolab Guinea Pig
	Mini-Pig	5080 Laboratory Mini-Pig Starter Diet; 5L80 Laboratory Mini-Pig HF Grower Diet; 5081 Laboratory Mini-Pig Grower Diet; 5082 Laboratory Mini-Pig Breeder Diet; 5084 Laboratory Porcine Grower Diet; 5P94 Prolab Mini-Pig Diet
	Dog	5006 Laboratory Canine Diet; 5007 Certified Laboratory Canine Diet; 5L18 Laboratory High Density Canine Diet; 5P40 Prolab Canine 1600; 5P41 Prolab Canine 2000
	Cat	5003 Laboratory Feline Diet
	Ferret	5280 Ferret Diet; 5L14 High Density Ferret Diet
	Avian	5065 Laboratory Chick Diet S-G; 5070 Laboratory Cage Layer Diet
	Ruminant	5508 Rumilab Diet
	Primate	5037 & 5038 Monkey Diet Jumbo and Monkey Diet; 5040 New World Primate Diet; 5045 & 5047 High Protein Monkey Diet and Jumbo; 5048 Certified Primate Diet; 5049 & 5050 Laboratory Fiber-Plus Monkey Diet and Jumbo; 5052 Fiber-Balance Monkey Diet; 5K91 Certified HiFiber Primate; 5P46 Prolab Primate 18
	Rodents	2014 Protein Rodent Maintenance Diet (14%) 2014S Protein Rodent Maintenance Diet (14%) (Sterilizable) 2016 Protein Rodent Diet (16%) 2016S Protein Rodent Diet (16%) (Sterilizable) 2018 Protein Rodent Diet (18%) 2018S Protein Rodent Diet (18%) (Sterilizable)
	Rabbits	2030 Rabbit Diet 2031 High Fiber Rabbit Diet
	Guinea Pig	2040 Guinea Pig Diet 2041 High Fiber Guinea Pig Diet
Harlan Teklad PO Box 44220 Madison, WI 53744-4220 Toll Free: (800) 483-5523 Voice: (608) 277-2070 FAX: (608) 277-2066 www.Harlan.com/tekla/ global/index	Primates	2050 Protein Primate Diet (20%) 2055 Protein Primate Diet (25%) 2021 Protein Dog Diet (21%) 2025 Protein Dog Diet (25%) 2027 Protein Dog Diet (27%)
	Cats	2060 Cat Diet

TABLE 1.4 (continued)
Types and Sources of Commercial Laboratory Diets

Source	Species	Diet Types
The above-mentioned diets are standard diets. Harlan Teklad also provides services to custom-design diets such as the examples listed as follows:		
Purified		Mineral deficient
Vitamin deficient		Adjusted calories
Adjusted protein		Amino acid diet
Adjusted carbohydrate		Adjusted fat
Essential fatty acid deficient		Atherogenic
Rabbit diets with cholesterol		Basal mixes
Isoflavone reduced		Basal mixes
^a Pico diets are irradiated.		

TABLE 1.5
Nutritional Deficiencies of Laboratory Animals

Nutritional Deficiency	Species Affected	Symptom(s)
Vitamin A	All species	Night blindness, dryness and thickness of cornea and conjunctiva, skin lesions
Vitamin C	Primates and guinea pigs	Scurvy conditions, breakdown of connective tissues
Vitamin D	All species	Lameness, enlargement of long bones with softening and deformation of all bones
Vitamin E	All species	Weak muscles, poor growth, low reproduction
Vitamin K	All species (the guinea pig <i>may</i> be an exception)	Slow blood clotting time
Vitamin B ₁	All species	Gastrointestinal, nervous, cardiovascular symptoms
Vitamin B ₂	All species	Skin lesions or mucous membrane lesions, cardiac problems in dogs, collapse, coma
Nicotinic acid	All species	Skin, gastrointestinal, nervous symptoms, inflammation of the mouth in dogs
Vitamin B ₆	All species	Convulsions, nausea, dermatitis, anemia
Biotin	Mice (raw egg whites or sulfur drugs can result in a deficiency for any mammal)	Skin lesions
Folic acid	All species	Anemia, diarrhea in primates
Choline	All species	Weight loss, reduced reproduction and lactation
Vitamin B ₁₂	All species	Anemia
Calcium	All species	Lameness
Phosphorus	All species	Lameness
Magnesium	All species	Low blood pressure, nervous symptoms
Sodium	All species	Reduced growth, eye disturbances, low protein digestion
Chlorine	All species	Abnormal fluid and pH balances
Potassium	All species	Reduced appetite and growth
Iron	All species	Anemia
Copper	All species	Anemia, hair loss, dermatosis
Iodine	All species	Weak newborns, decreased basal metabolism rate
Cobalt	Guinea pig	Anemia

FOOD AND WATER REQUIREMENTS

Approximate daily food and water requirements for various species are presented in Table 1.6.

Most toxicology studies employ *ad libitum* feeding conditions in which animals are allowed to regulate their own

dietary intake to meet energy requirements. However, the use of *ad libitum* feeding for long-term rodent bioassays has recently received increased attention since it appears that this practice impacts longevity, carcinogenesis, and overall animal health.

TABLE 1.6
Approximate Daily Food and Water Requirements
for Various Species

Species	Daily Food Requirement	Daily Water Requirement
Mouse	3–6 g	3–7 mL
Rat	10–20 g	20–30 mL
Hamster	7–15 g	7–15 mL
Guinea pig	20–30 g ^a	12–15 mL/100 g
Rabbit	75–100 g	80–100 mL/kg
Cat	100–225 g	100–200 mL
Dog	250–1200 g	100–400 mL/day
Primate	40 g/kg ^a	350–1000 mL

^a Like humans, guinea pigs and nonhuman primates require a continuous supply of vitamin C (ascorbic acid) in the diet.

FASTING

Like humans, animals are often fasted in preparation for blood collection. Generally, fasting periods of 18–24 h may be safely utilized for most species. However, for mice, fasting periods of 18–24 h may lead to severe debility, dehydration, and even death. Therefore, it is recommended that fasting periods of no longer than 4–6 h be used for this species.

ANESTHESIA AND ANALGESIA*

GENERAL CONSIDERATIONS

As stated earlier, investigators using live animals must employ appropriate anesthetic, analgesic, and sedative agents when necessary to control pain and distress, unless use of such agents would interfere with the specific objectives of the research. If these agents are not used, both the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals* require that the procedures be directly supervised by the responsible investigator in accordance with all regulations and guidelines governing these situations. If pain-relieving procedures are not employed, the investigator must provide well-documented evidence demonstrating that the use of such agents would interfere with the results of the study.

CONTROLLED SUBSTANCES

To comply with these regulations, it is imperative that appropriate pain-relieving agents be available and that appropriate methods of administration and dosages be established. Because many pain-relieving agents are controlled substances, the use and handling of these agents are regulated by the Controlled Substances Act (84 Stat. 1242; 21 U.S.C. 801). This statute is specifically administered by the US Drug Enforcement Agency (DEA). Among other requirements,

users of controlled substances must ensure that drug supplies are adequately protected (stored in a secure cabinet or safe) and inventoried in accordance with the requirements of the statute.

RELEVANT DEFINITIONS

- *Analgesia*: The relief of pain without loss of consciousness.
- *Tranquilization*: A state of behavioral change in which the animal is relaxed, unconcerned by its surroundings, and often indifferent to minor pain.
- *Sedation*: Mild state of CNS depression in which the animal is awake, but calm.
- *Local anesthesia*: Loss of sensation in a limited area.
- *Regional anesthesia*: Insensibility in a larger but still limited area.
- *Preal anesthesia*: A state produced by the concomitant use of several drugs to decrease anxiety without producing excessive drowsiness, to facilitate smooth, rapid induction of general anesthesia without prolonging emergence, provide amnesia for the perioperative period while maintaining cooperation prior to loss of consciousness, relieve preoperative and postoperative pain, and minimize some of the undesirable effects of anesthesia, that is, salivation, bradycardia, and postanesthetic vomiting.
- *General anesthesia*: A state of controlled and reversible unconsciousness characterized by lack of pain (analgesia), lack of memory (amnesia), and relatively depressed reflex responses without affecting the animal's vital systems, that is, respiration and circulation.
- *Surgical anesthesia*: Generally referred to as a surgical plane of anesthesia representing Stage III, plane 2 of the classical stages and planes of anesthesia; a medium depth of anesthesia suitable for most surgical procedures.

* See Appendix for additional related information at the end of this chapter.

GENERAL PRINCIPLES REGARDING ANESTHESIA, ANALGESIA, AND TRANQUILIZATION

The health of the animal should be carefully evaluated before instituting any anesthetic, analgesic, or tranquilizing procedure, and the specific drug(s) selected should provide the minimal level of CNS depression necessary. In addition, before undertaking any procedure, the investigator should closely consider the effect of the technique on experimental objectives, including potential drug interactions and interferences with test substance(s) (e.g., competing metabolic pathways, etc.).

STAGES OF ANESTHESIA

Indicators of anesthesia are commonly divided into four classic stages based on the depth of consciousness, presence or absence of reflex reactions, and degree of CNS and physiological depression. Stage I is characterized by disorientation, normal or panting respiration (20–30 breaths/min), unchanged heart rate, centrally positioned eyeball, normal pupil size, pupillary response to light, good muscle tone, and the presence of all reflexes. Stage II is marked by “excitement” with possible struggling, vocalization, paddling, chewing, or yawning; irregular respiration with possible holding of breath or hyperventilation; increased heart rate; centrally positioned eyeball or possible nystagmus (rapid involuntary oscillation of eyeball), possible dilation of pupils; pupillary response to light; good muscle tone; and presence of all reflexes with some possibly exaggerated. These first two stages represent presurgical anesthetic depths. Stage III anesthesia is subdivided into four different “planes” of progressively deeper unconsciousness. In plane 1 (light anesthesia), respiration is regular with a rate of 12–20 breaths/min; pulse is strong (>90 beats per min [bpm]); the animal may respond with movement, eyeballs may be centrally positioned, or there may be nystagmus; pupil size is normal and responds to light; muscle tone is good and swallowing reflex is poor or absent and others present but diminished. In plane 2 (medium or surgical anesthesia), respiration may be shallow at 12–16 breaths/min; heart rate >90 bpm; heart and respiration rates may increase in response to surgical activity; eyeballs may be ventrally rotated; pupil size moderately dilated; pupillary light response sluggish; muscle tone relaxed and patellar; ear flick, palpebral and corneal reflexes may be present, but others absent. In plane 3 (deep anesthesia), respiration is shallow at <12 breaths/min; heart rate is 60–90 bpm with increased capillary refill time (CRT) and reduced strength of pulse; there is no response to surgical activity; eyeballs may be central or rotated ventrally; pupils are moderately dilated; pupillary light response is very sluggish; muscle tone greatly reduced; and all reflexes are diminished or absent. In plane 4 (overdose), respiration is jerky; heart rate <60 bpm with prolonged CRT and pale mucous membranes; there is no response to surgical activity; eyeballs are centrally positioned; pupils are widely dilated; pupillary light response is absent; muscle tone is flaccid; and there is no reflex activity. In Stage IV, the animal is moribund with loss of thoracic breathing, cardiovascular collapse, centrally

positioned eyeballs, absence of pupillary light response, flaccid muscle tone, and absence of all reflexes.

The characteristics of the various stages and planes of anesthesia may vary with the anesthetic agent used, the species of animal, and the condition of individual animals with regard to study-specific treatments prior to anesthesia.

METHODS OF ADMINISTRATION

Anesthetic agents are commonly administered by parenteral injection, inhalation, tracheal intubation, or topical application. For inhalant anesthetics, use of appropriate equipment (i.e., gas anesthesia machine) is highly recommended to help assure proper control of exposure. Masks or cones may be used with gas anesthesia machines to initially induce anesthesia, or to maintain animals at the desired level of anesthesia.

Injectable compounds may be administered by various routes (e.g., IV, IM, IP, or SC) for the purpose of preanesthesia, or to obtain a surgical level of anesthesia. However, in all cases, dosages and drugs must be calculated carefully and animals closely monitored throughout the anesthesia procedure. In larger species, tracheal intubation is often used for administration of inhalant anesthetics because this method allows for oxygen administration and forced ventilation, if necessary.

When selecting an anesthetic agent and method of administration, the investigator must consider several factors, including the species, age, type and duration of surgery, available equipment, and personal knowledge. Of course, all procedures involving anesthesia and surgery must be supervised by a qualified veterinarian.

COMMONLY USED ANESTHETIC, ANALGESIC, AND TRANQUILIZING AGENTS

Some of the more commonly used anesthetic, analgesic, and tranquilizing agents are briefly described in the following list.

- *Atropine sulfate*: Anticholinergic agent often used as a preanesthetic to help decrease salivation, promote bronchodilation, prevent vagally induced bradycardia and reduced cardiac output, and reduce gastrointestinal activity.
- *Acepromazine maleate*: A phenothiazine sedative with antiemetic, antidysrhythmic, and antihistaminic properties.
- *Chlorpromazine hydrochloride*: This phenothiazine derivative potentiates barbiturate anesthesia.
- *Diazepam (Valium)*: Schedule IV drug with anti-convulsant and muscle relaxation properties.
- *Narcotic agents*: These agents produce hypnotic and analgesic effects, with resulting depression of cardiovascular and thermoregulatory systems (e.g., morphine, meperidine, etorphine [M99], and fentanyl).
- *Morphine*: May cause atropine-sensitive bradycardia and adverse gastrointestinal disturbance.

- *Meperidine (Demerol)*: Usually preferred over morphine because it produces fewer adverse side effects.
- *Fentanyl*: A potent short-acting narcotic used in Innovar-Vet (see following paragraphs).
- *Etorphine hydrochloride (M99)*: Commonly used to immobilize zoo animals and wild game.
- *Innovar-Vet*: Combination of narcotic analgesic fentanyl (0.4 mg/mL) and tranquilizer droperidol (20 mg/mL) which produces good analgesia and muscle relaxation.
- *Rompun (Xylazine)*: Nonnarcotic sedative and analgesic muscle relaxant with a wide margin of safety.
- *Ketamine hydrochloride (Vetalar)(Ketaset)* (Changed to a Schedule III drug by the DEA in August, 1999): Dissociative anesthetic agent that produces a state of chemical restraint and anesthesia. Reflexes remain intact. Excessive salivation may be controlled with atropine. Ketamine hydrochloride has a wide margin of safety and relatively short duration and recovery time, with minimal adverse side effects.
- *Medetomidine hydrochloride (Domitor)*: A synthetic α_2 -adrenoreceptor agonist which produces sedation and analgesia for clinical and minor surgical procedures not requiring muscle relaxation. Domitor can be reversed with *Atipamezole hydrochloride (Antisedan)*.
- *Pentobarbital sodium (Nembutal)*: Long-acting barbiturate with a small margin of safety. Produces severe CNS depression and general anesthesia with increasing dose.
- *Thiamylal sodium (Surital)*: Short-acting barbiturate (approximately 15–30 min).
- *Chloralhydrate*: A hypnotic Schedule IV drug with a narrow margin of safety and weak analgesic properties.
- *Diethylether*: Inhalant anesthetic has so many shortcomings that it should not be used. Although it provides good analgesia and muscle relaxation, vapors irritate the respiratory mucosa, and it is EXTREMELY FLAMMABLE AND EXPLOSIVE.
- *Halothane (Flurane)*: This highly volatile inhalant anesthetic produces reasonably good analgesia and muscle relaxation, but is a potent cardiovascular depressant. A vaporizer is essential to produce precise concentrations.
- *Methoxyflurane (Metafane)*: Nonexplosive inhalant anesthetic of relatively low volatility that produces good analgesia and muscle relaxation. Produces cardiovascular and respiratory depression.
- *Isoflurane (AErrane®)*: Nonflammable, nonexplosive general inhalation anesthetic agent. Produces profound respiratory depression. Increasing depth of anesthesia may increase hypotension and respiratory depression.
- *Nitrous oxide*: Potent inhalant anesthetic which is nonirritating, nonexplosive, and often used in conjunction with other agents.

Dosages and routes of administration of several commonly used anesthetic agents are presented in Table 1.7.

TABLE 1.7
Typical Routes and Dosages of Several Sedative, Analgesic, and Anesthetic Agents^a

Agents	Dosage and Route in Species						
	Mouse	Rat	Hamster	Guinea Pig	Rabbit	Dog	Primate
Chlorpromazine (mg/kg)	3–35 (IM) 6 (IP)	1–20 (IM) 4–8 (IP)	0.05 (IM)	5–10 (IM)	10–25 (IM)	1–6 (IM) 0.5–8 (PO)	1–6 (IM)
Promazine (mg/kg)	0.5 (IM)	0.5–1 (IM)	0.5–1 (IM)	0.5–1 (IM)	1–2 (IM)	2–4 (IM)	2–4 (IM)
Acepromazine (mg/kg)	—	—	—	—	1 (IM)	0.5–1 (IM) 1–3 (PO)	0.5–1 (IM)
Meperidine (mg/kg)	60 (IM) 40 (IP)	44 (IM) 50 (IP) 25 (IV)	2 (IM)	1 (IP) 2 (IM)	10 (IV)	0.4–10 (IM)	3–11 (IM)
Innovar-Vet (mL/kg)	0.05 (IM)	0.13–0.16 (IM)	—	0.08–0.66 (IM)	0.2–0.3 (IM)	0.13–0.15 (IM)	0.05 (IM)
Ketamine (mg/kg)	25 (IV) 25–50 (IP) 22 (IM)	25 (IV) 50 (IP) 22 (IM)	40 (IM) 100 (IP)	22–64 (IM)	22–44 (IM)	—	5–15 (IM)
Pentobarbital (mg/kg)	35 (IV) 40–70 (IP)	25 (IV) 40–50 (IP)	50–90 (IP)	24 (IV) 30 (IP)	25 (IV) 40 (IP)	30 (IV)	25–35 (IV)
Thiopental (mg/kg)	25–50 (IV)	40 (IM) 25–48 (IP)	—	55 (IM) 20 (IP)	25–50 (IV)	16 (IV)	25 (IV)

Note: See Appendix for additional related information on anesthetics at the end of this chapter.

^a Drugs and dosages presented are to serve only as a guideline. Selection and administration of specific agents and dosages should be supervised by a qualified veterinarian.

SPECIES PECULIARITIES AND CONTRAINDICATIONS

Mouse

- Use of chloroform in the mouse can cause renal tubular calcification and/or necrosis, especially in males. The DBA/2 mouse strain is particularly susceptible to these effects.

Rat

- Use of methoxyflurane is contraindicated in the Fischer 344 rat because this inhalant anesthetic may produce a diabetes-like syndrome in this strain.

Guinea Pig

- Intramuscular injection of Innovar-Vet should be avoided in guinea pigs because this can produce severe tissue necrosis.
- Repeated exposure to halothane in the guinea pig can produce hepatotoxicity. In addition, guinea pigs routinely hold their breath when first exposed to the irritating vapors (e.g., from halothane or chloroform). Thus, methoxyflurane is considered a safer alternative for this species.
- The larger cecum of the guinea pig can act as an anesthetic reservoir.

Rabbit

- A combination of 35 mg ketamine with 5 mg xylazine/kg given IM is a safe and effective method of anesthesia in the rabbit (20–75 min).
- The rabbit possesses a unique hypnotism/immobilization reflex.
- Like the guinea pig, the large cecum of the rabbit may act as an anesthetic reservoir.

Cat

- The use of morphine is contraindicated in the cat.

Primate

- Tranquilizers should never be used as the sole method of restraint for primates.
- The most commonly used immobilization agent for primates is 10–40 mg/kg ketamine given IM.
- For general surgical procedures, inhalation anesthesia is best, with 0.1 mg/kg atropine sulfate to control salivation.

EUTHANASIA

Over the years, a number of acceptable and effective methods have been developed and utilized to induce euthanasia in various species. A detailed discussion of these and other euthanasia methods may be found in the 2007 AVMA Guidelines on Euthanasia.⁶ The ultimate goal of euthanasia is to induce humane death, without causing unnecessary anxiety, pain, or distress to the animal. To achieve this, the euthanasia method must produce rapid CNS depression and

insensitivity to pain to minimize potential stress and/or anxiety which might otherwise occur before unconsciousness. Thus, when employed in an appropriate manner, a good euthanasia method will induce CNS depression and rapid unconsciousness, followed by respiratory or cardiac arrest, and subsequent loss of brain function.

Unfortunately, there are a number of euthanasia techniques that, although generally recognized as humane, possess a high degree of intrinsic unpleasantness. Thus, researchers are challenged to select euthanasia techniques that (1) induce humane death without causing pain or distress to the animals, (2) do not negatively impact on experimental objectives and postmortem evaluations, and (3) do not produce an unnecessary level of unpleasantness for those involved. With regard to the last consideration, it should be emphasized that some intrinsically unpleasant methods of euthanasia are nonetheless humane. Before using these “unpleasant methods,” researchers are encouraged to educate personnel concerning the lack of an acceptable alternative method, and to have appropriate and detailed documentation supporting the need for the particular euthanasia method selected. Finally, it is imperative that individuals involved in performing any euthanasia procedure be properly trained and possess a demonstrated proficiency in the particular technique before undertaking the procedure with any animal.

MODES OF ACTION

Euthanasia agents produce death by three primary mechanisms: (1) direct or indirect hypoxia, (2) direct depression of neurons essential for life functions, or (3) physical disruption of brain activity via destruction of essential neuronal components. Agents that induce death by direct or indirect hypoxia should produce unconsciousness before loss of motor activity to ensure a painless and distress-free death. Agents that cause muscle paralysis without unconsciousness are therefore unacceptable as the sole method of euthanasia (e.g., curare, succinylcholine, etc.). Agents that produce unconsciousness and death by direct depression of neurons in the brain may produce an initial stage of “excitement” during which muscle contraction and vocalization may occur. These responses should not be regarded as indicators of distress because they do not seem to be purposeful. Death from these agents is attributable to direct depression of respiratory centers and/or cardiac arrest.

When properly implemented, physical disruption of brain activity (e.g., by concussion), direct destruction of the brain (e.g., by penetrating captive bolt), and electrical depolarization of neurons (e.g., by electrocution) are effective methods for the rapid induction of unconsciousness and death. However, these methods are often aesthetically objectionable for those involved. Exaggerated muscle activity may follow unconsciousness from these methods; however, the animal is not thought to experience pain or distress in the unconscious state.

EUTHANASIA METHODS AND AGENTS

The selection of a particular euthanasia method requires consideration of several factors, including the age and species to be euthanized; ability of the method/agent to induce unconsciousness and death without causing pain or distress; training and skill of personnel; reliability and irreversibility of the method; safety of personnel; and compatibility of

the method with experimental objectives and end points. Euthanasia agents and methods which are currently considered acceptable or “conditionally acceptable” by the AVMA Guidelines are presented for several common laboratory species in Table 1.8. The characteristics and modes of action of these agents/methods are summarized in Table 1.9 and briefly described in the following sections.

TABLE 1.8
Acceptable and “Conditionally Acceptable” Methods for Euthanasia of Several Common Laboratory Species

Species	Agents/Methods ^a	
	Acceptable	Conditionally Acceptable
Cats	Inhalant anesthetics, CO, CO ₂ , barbiturates, KCl with prior general anesthesia	N ₂ , Ar
Dogs	Inhalant anesthetics, CO, CO ₂ , barbiturates, KCl with prior general anesthesia	N ₂ , Ar, electrocution, penetrating captive bolt
Swine	CO ₂ , barbiturates, KCl with prior general anesthesia, penetrating captive bolt	Inhalant anesthetics, CO, chloral hydrate (IV after sedation), gunshot, electrocution, blow to head (<3 weeks of age)
Rabbits	Inhalant anesthetics, CO, CO ₂ , barbiturates, KCl with prior general anesthesia	N ₂ , Ar, cervical dislocation, decapitation, penetrating captive bolt
Rodents and other small animals	Inhalant anesthetics, CO, CO ₂ , barbiturates, KCl with prior general anesthesia	Methoxyflurane, ether, N ₂ , Ar, cervical dislocation (for rats <200 g), decapitation
Nonhuman primates	Barbiturates	Inhalant anesthetics, CO, CO ₂ , N ₂ , Ar

Source: AVMA, *AVMA Guidelines on Euthanasia*, AVMA, Schaumburg, IL, 2007.

^a See Table 1.9 for other conditions and requirements of “acceptable” and “conditionally acceptable” euthanasia methods.

TABLE 1.9
Summary of the Characteristics of Several Euthanasia Methods

Euthanasia Method	Classification	Mechanism of Action	Species	Effectiveness	Personnel Safety
Inhalant anesthetics	Acceptable	Hypoxia due to depression of vital centers	Small animals such as rats, mice, hamster, and guinea pigs via chamber administration	Moderately rapid onset of anesthesia; initial excitation may occur	Minimize exposure to personnel by scavenging or venting
Carbon dioxide	Acceptable	Hypoxia due to depression of vital centers	Small animals such as rats, mice, hamsters, and guinea pigs via chamber administration	Effective in adult animals; may be prolonged in immature and neonatal animals	Minimal hazard
Carbon monoxide	Acceptable	Hypoxia due to inhibition of O ₂ -carrying capacity of hemoglobin	Most small species including dogs, cats, and rodents	Effective and acceptable with proper equipment and operation	Extremely hazardous; difficult to detect
Barbiturates	Acceptable	Hypoxic due to depression of vital centers	Most species	Highly effective when administered appropriately	Safe, except human abuse potential of controlled substances(s)
Inert gasses (N ₂ , Ar)	Conditionally acceptable	Hypoxic hypoxemia	Cats, small dogs, rodents, rabbits, and other small species	Effective, but other methods are preferable; acceptable only if animal is heavily sedated or anesthetized	Safely used in ventilated area

TABLE 1.9 (continued)
Summary of the Characteristics of Several Euthanasia Methods

Euthanasia Method	Classification	Mechanism of Action	Species	Effectiveness	Personnel Safety
Cervical dislocation	Conditionally acceptable	Hypoxia due to disruption of vital centers, direct depression of brain	Mice, rats <200 g, and rabbits <1 kg	Effective and irreversible; requires training, skill, and IACUC approval; aesthetically displeasing	Safe
Decapitation	Conditionally acceptable	Hypoxia due to disruption of vital centers, direct depression of brain	Rodents and small rabbits	Effective and irreversible; requires training, skill, and IACUC approval; aesthetically displeasing	Potential injury due to guillotine

Inhalant Agents

The suitability of a particular inhalant agent depends on whether the animal experiences distress before loss of consciousness. Additional considerations common to all inhalant agents are listed as follows:

- In general, unconsciousness is more rapid and euthanasia is more humane when the animal is rapidly exposed to a high concentration of the agent.
- Most inhalant agents are hazardous to humans. Therefore, appropriate safety precautions must be followed to ensure personnel safety.
- Compared to adult animals, neonates are often more resistant to the effects of inhalant agents due apparently to increased resistance to hypoxia.

Inhalant anesthetics, such as ether, halothane, methoxyflurane, isoflurane, and enflurane, have been used in overdose for euthanasia of smaller animals. Exposure is usually accomplished using a small chamber (e.g., a bell jar) containing cotton or gauze soaked with the inhalant anesthetic agent. The use of suspended wire flooring in the chamber allows equilibration of the chamber atmosphere, while avoiding direct contact by the animal with the irritating liquid anesthetic.

Advantages

- Inhalant anesthetics are useful for euthanasia of small animals in which venipuncture may be difficult.
- Halothane, enflurane, isoflurane, and methoxyflurane are nonflammable and nonexplosive under conditions of routine use.

Disadvantages

- Struggling and anxiety may develop during induction due to irritating vapors.
- Ether is extremely flammable and explosive.
- Inhalant anesthetic vapors may be harmful to humans, particularly to the developing conceptus during the early stages of pregnancy.

Recommendations

- Chamber administration of the inhalant anesthetics listed earlier is acceptable for euthanasia of small animals such as rats, mice, hamsters, and guinea pigs.

However, if possible, use of ether should be avoided because it is extremely flammable and potentially explosive. In addition, appropriate safety precautions should be used with all inhalant anesthetic agents to avoid exposure of laboratory personnel.

Carbon Dioxide

Carbon dioxide (CO₂) is a nearly odorless, nonflammable, and nonexplosive gas that has been used extensively as an inhalant euthanasia agent for a number of species.

Advantages

- CO₂ produces rapid depressant and anesthetic effects.
- CO₂ may be obtained in compressed cylinders.
- CO₂ is inexpensive, nonflammable, and nonexplosive; it does not pose a particular safety hazard to personnel under conditions of normal use.
- CO₂ does not distort cellular architecture.

Disadvantages

- There are no major disadvantages concerning the use of CO₂ as a euthanasia agent. However, it should be noted that because CO₂ is heavier than air, incomplete chamber filling may permit taller or climbing animals to avoid exposure.

Recommendations

- Chamber administration of CO₂ is an effective and often desirable method of euthanasia for small animals such as rats, mice, hamsters, and guinea pigs, provided that the chamber is not overcrowded. CO₂ is not recommended for larger animals such as rabbits, cats, and dogs, because these species may exhibit signs of distress before the onset of anesthesia and narcosis. CO₂ is best provided from pressurized tanks that allow precise regulation of CO₂ inflow. Effective exposure conditions for the most smaller species are a CO₂ concentration of 70% (i.e., 70% CO₂ and 30% O₂), and a flow rate which displaces approximately 20% of the chamber volume per minute. Do not prefill the euthanasia chamber with CO₂. Start with room air, and then slowly fill the chamber with CO₂ over several minutes.

Carbon Monoxide

Carbon monoxide (CO) is a colorless, odorless gas that is flammable and potentially explosive at concentrations above 10%. In humans and animals, CO acts as a cumulative poison by combining with hemoglobin and blocking the uptake of oxygen by red blood cells, leading to fatal hypoxemia. Although CO has been shown to induce unconsciousness in animals with minimal discernible discomfort, the many dangers associated with CO outweigh its routine use in most laboratory settings. Nonetheless, CO inhalation is an acceptable method for euthanasia of many species, including dogs and cats, provided that compressed CO is used and the following precautions are taken:

- Personnel must be thoroughly instructed in the use of CO and its associated hazards and limitations.
- The CO source and chamber must be in a well-ventilated area, preferably outdoors.
- The chamber must be well lit and have viewing ports that allow observation of the animals.
- The CO flow rate must be adequate to rapidly achieve a CO concentration of at least 6%.
- If the CO chamber is inside, CO monitors must be placed in the room to warn personnel of hazardous CO concentrations.

Inert Gases

Inert gases such as nitrogen and argon are colorless, odorless, nonflammable, and nonexplosive gases that have been used to induce euthanasia by hypoxemia. Although these gases are readily available and minimally hazardous to personnel, their use requires prior sedation or anesthesia of the animal to avoid discernible hypoxemia and ventilatory stimulation which commonly precede death and are obviously distressing. In addition, when preanesthesia is used, the time to death is often delayed. Consequently, inert gases should be used for euthanasia only when animals have been heavily sedated and chamber oxygen concentrations of less than 2% can be rapidly achieved.

Chloroform

Chloroform presents a significant hazard due to its known potent hepatotoxicity and suspected carcinogenicity in humans. Therefore, chloroform is not recommended for euthanasia.

Barbiturates and Barbiturate Combination Drugs

There are several commercially available euthanasia products that are formulated to include a barbituric acid derivative such as phenobarbital and local anesthetic agent(s). These products are often categorized as Schedule III drugs, making them somewhat easier to obtain and store compared with Schedule II drugs such as phenobarbital. These agents are acceptable and effective for euthanasia when properly used. Combination drugs containing neuromuscular blocking agents are not acceptable for euthanasia.

Chloral Hydrate

Chloral hydrate causes death by hypoxemia resulting from depression of the respiratory center. However, because this

depression is slow, it may be preceded by aesthetically objectionable symptoms such as muscle spasms, gasping, and vocalization. Thus, chloral hydrate is not considered acceptable for euthanasia of dogs, cats, or other small animals. Chloral hydrate is conditionally acceptable for the euthanasia of large animals when administered intravenously following prior sedation.

T-61 Euthanasia Solution

T-61, an injectable nonbarbiturate, nonnarcotic combination of three drugs, is no longer manufactured or commercially available in the United States, but is available in Canada and other countries. T-61 should only be administered by the intravenous route with monitoring of the injection rate.

Unacceptable Injectable Agents

The following injectable agents are considered unacceptable for euthanasia when used alone: strychnine, nicotine, caffeine, magnesium sulfate, potassium chloride, cleaning agents, solvents, disinfectants, and other toxins or salts, and all neuromuscular blocking agents.

Cervical Dislocation and Decapitation

Physical methods of euthanasia such as cervical dislocation and decapitation are considered by most to be aesthetically displeasing. However, when properly used by skilled personnel, these methods may cause less fear and anxiety, and may be more rapid, painless, and humane than other methods of euthanasia. In the laboratory, cervical dislocation and decapitation may be useful euthanasia techniques for small animals when other methods or agents may interfere with experimental objectives and results. However, before using these methods, it is imperative that personnel are properly trained and experienced, and that approval is obtained from the Institutional Animal Care and Use Committee.

Recommendations

Physical euthanasia methods such as cervical dislocation and decapitation are recommended only when scientifically justified and when other acceptable methods have been clearly ruled out. Use of these procedures must be preapproved by the Institutional Animal Care and Use Committee. Animals should be sedated or unconscious before using these techniques, if practical. When properly performed, cervical dislocation is considered humane for poultry, mice, rats weighing less than 200 g, and rabbits weighing less than 1 kg.

Verification of Death

Regardless of the specific euthanasia method used, it is imperative that death be verified by examining the animal for cessation of vital signs. Of course, the specific means for confirming death requires professional judgment and training.

SOURCES OF LABORATORY ANIMALS

There are a number of reliable sources from which laboratory animals may be purchased. Company names, addresses, telephone numbers, and available species from several suppliers are presented in Table 1.10.

TABLE 1.10
Names, Addresses, and Phone Numbers of Several Animal Suppliers

Facility	Guinea															Contact Information
	Cats	Cattle	Chinchillas	Dogs	Gerbils	Pigs	Hamsters	Mice	Opposum	Primates	Rabbits	Rats	Sheep	Swine	Woodchucks	
Alder Ridge Farms, Inc., PA				x											(570) 727-3459	
Animal Biotech Industries, PA													x	x	(215) 766-7413 animalbiotech.com	
Archer Farms, MD													x	x	(410) 879-4110 archerfarmsinc.com	
B&K Universal, Inc., UK				x	x	x	x	x		x		x			(01964) 527555 bku.com	
Barton's Farms and Biologicals, NJ				x									x	x	(908) 637-4427	
Charles River Laboratories, MA					x	x	x	x		x	x	x			(800) 522-7287 criver.com	
CLEA Japan/Pegasus, NJ								x		x		x			(609) 737-3961 straube.com	
Covance, PA				x						x	x				(717) 336-4921 covance.com	
Harlan Sprague Dawley, Inc., IN				x		x	x	x			x	x			(800) 793-7287 harlan.com	
Hilltop Lab Animals, Inc., PA						x		x				x			(724) 887-8480 hilltoplabs.com	
Ridgland Farms, Inc., IN				x											(877) 437-8670	
Jackson Laboratory, ME								x							(800) 422-6423 jaxmice.jax.org	
Liberty Research, Inc., NY	x			x											(607) 565-8131 lirresearch.com	
Marshall BioResources, NY				x										x	(315) 587-2295 marshallbio.com	
Moulton Chinchilla Ranch, MN			x												(507) 288-6334	
Northeastern Wildlife, ID									x						(208) 689-9172. northeasternwildlife.com	
Robinson Services, Inc., NC											x		x		(336) 940-2550 rsbiotech.us	
															(continued)	

(continued)

TABLE 1.10 (continued)
Names, Addresses, and Phone Numbers of Several Animal Suppliers

Facility	Guinea														Contact Information
	Cats	Cattle	Chinchillas	Dogs	Gerbils	Pigs	Hamsters	Mice	Opposum	Primates	Rabbits	Rats	Sheep	Swine	
Sage® Labs (Sigma-Aldrich)								x				x			800-325-3010 sageresearchmodels.com
Simonsen Laboratories, Inc., CA							x	x				x			(408) 847-2002 simlab.com
Sinclair Research Center, Inc., MO	x													x	(573) 387-4400 sinclairresearch.com
Taconic Farms, Inc., NY								x				x			(518) 537-5200 taconic.com
Thomas D. Morris, Inc., MD		x											x	x	(410) 356-6780 thomasmorris.com
Three Springs Scientific, PA										x					(215) 257-6055
Western Oregon Rabbit Co., OR															(541) 929-2245
Worldwide Primates, Inc.										x					(305) 378-9585 www.primates.com

SPECIES DATA

The following sections provide some general information concerning the husbandry and biology of several species commonly used in research. See Chapters 2, 6, 10 and 18 for additional detailed information.

MOUSE (*MUS MUSCULUS*)

Stocks and Strains

There are a variety of mouse stocks and strains which are available from commercial sources. Inbred strains are produced by 20 or more consecutive generations of brother × sister matings, with the primary objective of reducing genetic variability by increasing homozygosity at genetic loci. This results in a high degree of uniformity in the physical and physiological traits of the various inbred strains.

Outbred strains are produced through the mating of totally unrelated individuals. This frequently results in the production of offspring that show more vigor than the parental animals in terms of growth, survival, and fertility. The offspring of such matings (F1 hybrids) are heterozygous at all loci in which the parental animals differed. These F1 hybrids can be reproduced only from the designated parental strains.

Random breeding is a mating technique in which mating is undertaken with animals from the same stock, but without regard to genetic background. The primary purpose of this technique is to preserve genetic variability in the stock. Some of the more common mouse stocks and strains are briefly described in Table 1.11.

Handling and Restraint

Handling and restraint of the mouse require training and experience because of the mouse's small size and agile movement. The mouse is usually picked up by the tail, placed on a secure surface such as the forearm or table top, and then restrained by gently grasping the loose skin behind the neck and over the back, while maintaining a grip on the tail. The handler must use a firm but gentle grip to minimize twisting and movement of the animal that could potentially result in self-injury. Excessive pressure or force during handling and restraint could easily result in spinal separation or other injury to the mouse. Furthermore, extra care should be exercised when removing the mouse from a cage with a wire floor as the animal will forcefully grip the cage bottom to avoid removal.

Housing

Mice may be housed individually or with several animals per cage in plastic shoebox-type caging or suspended stainless steel caging. When housed in plastic shoeboxes, mice should be provided with some type of bedding material such as processed hardwood chips or ground corncobs. Fresh water may be provided using water bottles or via an automatic watering system. However, when using an automatic watering system, the line pressure must be low enough to allow the animal to easily activate the sipper mechanism without receiving a frightening high-pressure “squirt” in the face. In addition, whenever possible, gang housing of adult males should be avoided, because male mice will form a pecking order when placed together, and serious injury or death may result due to extensive fighting between cage mates. Minimum cage space requirements for mice are presented in Table 1.12.

Environmental Conditions

Environmental conditions recommended for mice by the *Guide for the Care and Use of Laboratory Animals* are as follows:

Room temperature: 68°F–79°F, 20°C–26°C

Relative humidity: 30%–70%

Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges to avoid extremes and large fluctuations in these environmental variables. The *Guide* does not specify any particular lighting cycle for mice; however, a 12 h light/12 h dark cycle is used routinely for this species.

Physical and Physiological Parameters

Physical and physiological parameters of laboratory mice are listed in Table 1.13.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Mice (Table 1.14)

Diseases

Various diseases and adverse health conditions of laboratory mice are identified and briefly described in Table 1.15.

TABLE 1.11
Common Stocks and Strains of Laboratory Mice

Strain	Description
CD-1 Mice	A multipurpose outbred albino strain descended from “Swiss” mice. Variants include the athymic CD-1 Nude Mouse often used in tumor xenograft research.
CF-1 Mice	Inbred from albino mice of likely wild origin for over 20 generations and then outbred to produce the current stock. A multipurpose model often used in infectious disease research.
Swiss–Webster Mice	A multipurpose outbred albino strain from selective inbreeding of Swiss mice by Dr. Leslie Webster.
SKH1 (Hairless) mice	Outbred strain that originated from an uncharacterized strain. Although the SKH-1 mouse resembles the nude mouse in appearance, this strain is euthymic and immunocompetent. Research uses include wound healing, dermatology, and UV-induced tumorigenesis.
BALB/c mice	An inbred albino strain developed originally by H.J. Bagg (Bagg albino). Frequently used for monoclonal antibody production, hybridoma development, and infectious disease research. Variants include the BALB/c Nude Mouse used for tumor xenografts and tumor biology.
C3H mice	An inbred agouti strain developed originally from the cross of a “Bagg albino” female and a DBA male. Used for oncology, neurologic research, and to study spontaneous retinal degeneration.
C57BL/6 mice	An inbred black strain developed originally by C.C. Little. A multipurpose model often used as the parental background strain for transgenic and knockout models. Variants include the Pound Mouse, isolated from a C57BL/6 colony at Charles River and used in research on diabetes and obesity.
DBA/2 mice	The oldest of all inbred mouse strains, the DBA/2 mouse is an inbred nonagouti, dilute brown strain developed originally by C.C. Little. A general model often used in immunology and experimental epilepsy.
FVB mice	An inbred albino strain derived originally from an outbred Swiss colony. Used primarily for transgenic and knockout model development.
AKR mice	An inbred albino strain originally developed by Furth as a high leukemia strain. Used for cancer, atherosclerosis, and metabolic research.
B6C3F1 mice	A hybrid agouti strain from female C57BL/6N × male C3H/He mice. Used by the National Toxicology Program as the mouse strain for carcinogenicity studies.
<i>Notes:</i> The two transgenic lines that follow (i.e., the rasH2 and the p53 mouse) have been commonly used as alternatives to the standard 2-year mouse carcinogenicity study. Not listed below, the Tg.AC mouse (carrier of an activated mouse H- <i>ras</i> oncogene) was used as an alternative to the 2-year mouse carcinogenicity study for dermal products but is now seldom used due to a high incidence of false positives.	
rasH2 mouse	The <i>ras</i> oncogenes are involved in approximately one-third of human cancers. The rasH2 mouse is produced on a CB6F1 mouse background and has the human c-Ha- <i>ras</i> oncogene in addition to its native murine Ha- <i>ras</i> proto-oncogene. Due to its sensitivity to both genotoxic and nongenotoxic compounds and its low spontaneous tumor incidence at 6 months, this transgenic model has become the primary replacement for the standard 2-year mouse carcinogenicity assay.
p53 ^{+/-} mouse	The p53 tumor suppressor gene, often mutated or deleted in human and rodent tumors, is critical to cell cycle control and DNA repair. The heterozygous version of the p53 model has one functional wild-type p53 allele and one inactivated allele, imparting sensitivity to the mutational and carcinogenic effects of genotoxic chemicals. The model tends to be rather insensitive, and most of the studies conducted in this model for regulatory purposes have yielded negative results.

TABLE 1.12
Minimum Cage Space Requirements for Mice^a

Body Weight (g)	Floor Area/Mouse in. ² (cm ²)	Cage Height in. (cm)
<10	6.0 (38.7)	5 (12.7)
10–15	8.0 (51.6)	5 (12.7)
15–25	12.0 (77.4)	5 (12.7)
>25	>15.0 (≥96.7)	5 (12.7)

^a As per the National Research Council of the National Academies, 2011.

TABLE 1.13
Physical and Physiological Parameters of Mice

Life span	1–2 years
Male adult weight	20–35 g
Female adult weight	20–35 g
Birth weight	1.0–1.5 g
Adult food consumption	3–6 g/day
Adult water consumption	3–7 mL/day
Male breeding age/weight	6–8 weeks/20–35 g
Female breeding age/weight	6–8 weeks/20–30 g
Placentation	Discoidal endotheliochorial
Estrus cycle	4–5 days (polyestrous)
Gestation period	19–21 days
Weaning age/weight	21 days/8–12 g
Average litter size	10–12 pups
Mating system(s)	1:1 or 1 male to multiple females
Adult blood volume	6%–7% of body weight
Maximum safe bleed	7–8 mL/kg
Red cell count	$7\text{--}12 \times 10^6/\text{mm}^3$
White cell count	$3\text{--}12 \times 10^3/\text{mm}^3$
Hemoglobin	13–17 g/dL
Hematocrit	40%–54%
Mean corpuscular volume	43–54
Mean corpuscular hemoglobin	13–18
Mean corpuscular hemoglobin concentration	31–34
Platelet count	$1000\text{--}1600 \times 10^3/\text{mm}^3$
Heart rate	300–600 beats/min
Respiration rate	90–180 breaths/min
Rectal temperature	37.5°C
Urine pH	6.0–7.5
Urine volume	1–3 mL/day
Chromosome number	$2n = 40$

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Williams, C.S.F., *Practical Guide to Laboratory Animals*, C.V. Mosby, St. Louis, MO, 1976; *LAMA Lines*, Newsletter of the Laboratory Animal Management Association, 4, September/October, 1988.

TABLE 1.14
Identification, Bleeding, Anesthesia, and Euthanasia Methods for Laboratory Mice

Identification methods
Ear tags
Ear punch, notch
Tail tattoo identification number
Subcutaneously implanted transponder
Bleeding methods
Orbital sinus
“Tail Nick”
Via heart after euthanasia
Anesthesia methods
Ketamine/xylazine, methoxyflurane, barbiturates,
pentobarbital (5 mg/100 g IP)
Euthanasia methods
Barbiturate overdose
CO ₂ inhalation
Cervical dislocation

TABLE 1.15
Various Diseases and Adverse Health Conditions of Laboratory Mice

Disease/Health Condition	Etiology, Clinical Signs, Symptoms, and/or Pathology
Tyzzer's	Caused by the bacterium <i>Clostridium piliforme</i> , an obligate intracellular organism that causes enterohepatic disease in many domestic and laboratory animal species; usually subclinical but immunosuppressant drugs may precipitate epidemics; signs include diarrhea, poor coat, and sudden death in young; focal necrosis in liver and inflammation of ileum may be seen at necropsy.
Murine respiratory mycoplasmosis (MRM)	Caused by bacterium <i>Mycoplasma pulmonis</i> ; relatively common chronic disease characterized by inflammation of respiratory tract and middle ear; signs in mice include chattering and dyspnea; lesions include bronchitis, bronchopneumonia, rhinitis, and otitis media. Other mycoplasma organisms infect mice, affecting the reproductive and central nervous systems.
Klebsiellosis	Caused by bacterium <i>Klebsiella pneumoniae</i> ; nonspecific signs include dyspnea, sneezing, cervical lymphadenopathy, inappetence, hunched posture, and rough coat; cervical, pharyngeal, renal, and hepatic abscesses; granulomatous pneumonia.
Staphylococcosis	Caused by bacterium <i>Staphylococcus aureus</i> ; normal inhabitant of the skin; may cause skin and facial abscesses in nude mice.
Pseudotuberculosis	Caused by bacterium <i>Corynebacterium kutscheri</i> ; infection usually inapparent but may cause nasal/ocular discharge, dyspnea, arthritis, or skin abscesses; focal caseous abscesses in liver, lungs, kidneys, and lymph nodes.
<i>Helicobacter hepaticus</i>	Causes chronic hepatitis and may be associated with increased incidence of hepatic neoplasms; mice may be infected with <i>H. bilis</i> , <i>H. muridarum</i> , <i>H. rappini</i> , and possibly others. <i>H. muridarum</i> may be associated with chronic gastritis, and one or more of the previously mentioned may be associated with chronic enterocolitis in immunodeficient mice.
Sendai virus	Caused by <i>Paramyxovirus</i> ; clinically inapparent chronic infection or clinically apparent acute infection; variable signs may include chattering, mild respiratory distress, prolonged gestation, poor growth, and death in young; concurrent pulmonary infections may occur.
Pneumonia virus of the mouse (PVM)	Caused by <i>Pneumovirus</i> ; common in laboratory rodents worldwide; subclinical in euthymic rodents.
K virus (Kilham virus of mice)	Caused by <i>Parvovirus</i> ; wild mice are natural hosts; natural infection is subclinical.
Epizootic diarrhea of infant mice (EDIM)	Caused by a rotavirus virus; mustard colored feces; rectal impaction follows intestinal inflammation.
Reovirus-3	Natural infections are usually subclinical and have little significance for most studies.
Murine hepatitis virus (MHV)	Caused by <i>Coronavirus</i> (25 different strains isolated); mice are natural hosts; clinically apparent infection in naive infant mice; diarrhea with high mortality may occur.
Mouse pox (ectromelia)	Caused by ectromelia virus; natural transmission due to direct contact and fomites; clinical manifestations may include variable mortality, facial edema, swelling of feet, and necrotic amputation of limbs or tail; necrosis of liver, spleen, and lymphoid tissue in acute disease.
Lymphocytic choriomeningitis	Caused by <i>Arenavirus</i> ; may cause significant zoonotic infection in those working with transplantable rodent tumors and rodent cell lines; wild mice are principal reservoirs; only infected mice and hamsters are known to transmit virus; natural infection of adult mice ranges from inapparent to severe disease with high mortality; clinical symptoms in humans are usually flu-like (see zoonoses).
Murine cytomegalovirus	Common subclinical infection of submaxillary salivary glands of wild mice; infrequent natural infections in laboratory mice; used as an animal model for human cytomegalovirus.
Mouse thymic virus	Caused by a Herpes virus, natural infections are subclinical; wild and laboratory mice are hosts; prevalence in mouse stocks is unknown; characteristic lesion is lymphoid necrosis in thymus, nodes, and spleen.
Polyomavirus	Highly contagious but of limited significance as natural infection of mice; major importance is as model for viral carcinogenesis and cell transformation; prevalence is poorly understood.
Minute virus of mice (MVM)	Caused by <i>Parvovirus</i> ; wild and laboratory mice are natural hosts; highly contagious but natural infections are inapparent and not known to produce disease.
Theiler's mouse encephalomyelitis	Caused by <i>Picornavirus</i> ; laboratory mice and rats are natural hosts, but infection is probably rare; predominant lesion is poliomyelitis.

TABLE 1.15 (continued)**Various Diseases and Adverse Health Conditions of Laboratory Mice**

Disease/Health Condition	Etiology, Clinical Signs, Symptoms, and/or Pathology
<i>Pneumocystis carinii</i>	Caused by what molecular genetic data confirm to be a fungus. Not pathogenic for immunocompetent hosts; steroids, low-protein diets, and immunodeficient genotypes can precipitate expression of varying degrees of interstitial pneumonia.
Protozoan parasites	<i>Cryptosporidium muris</i> , <i>C. parvum</i> ; <i>Eimeria</i> spp.; <i>Giardia muris</i> and <i>Spironucleus muris</i> can affect various levels of the gastrointestinal tract.
Fighting	Trauma due to fighting often results in morbidity and mortality in male mice housed together; fighting usually occurs at night; bite and scratch wounds often become infected; may be prevented by grouping males at weaning rather than later.
Hair chewing (barbering)	Alopecia in cage mates is most common in pigmented mice; early indication is loss of whiskers; alopecia of the muzzle, head, and trunk is common.
Ringtail	Condition of young rats and mice characterized by annular constriction and subsequent edema, necrosis, and sloughing of the tail; may be prevented by providing relative humidity $\geq 50\%$; much more common in rats than mice.

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, *Infectious Diseases of Mice and Rats*, National Academy Press, Washington, DC, 1991; Clarence, M.F., *The Merck Veterinary Manual*, 7th edn., Merck & Co., Rahway, NJ, 1991.

RAT (*RATTUS NORVEGICUS*)**Stocks and Strains**

The laboratory rat is derived from the wild brown or “Norway” rat. Outbred strains include the Sprague-Dawley, Wistar Han, and Long-Evans rats. Inbred strains include the Fischer 344, the Spontaneously Hypertensive rat, the Zucker Diabetic Fatty rat, the Lewis rat, and the Wistar Kyoto rat. Physiologically, a rat is similar to other single-stomached animals, except that rats do not possess a gall bladder. Common stocks and strains of rats that are available from several commercial suppliers are presented in Table 1.16.

Handling and Restraint

Laboratory rats can be handled easily if they are treated kindly. Frequent handling usually makes the rat even more gentle and easy to handle. Rats may be picked up by gently grasping the animal around the torso. If necessary, rats may be picked up by grasping the base of the tail and then immediately transferring the animal to a more stable position. However, when using this technique, it is important to grasp near the base and not the tip of the tail because this could easily injure the animal.

Housing

The rat is a most adaptive creature; however, this does not preclude the need for appropriate housing and husbandry standards. Like mice, rats may be housed individually or with several animals per cage in plastic shoebox-type caging or suspended stainless steel caging. When housed in plastic shoeboxes, rats should be provided with some type of bedding material such as processed hardwood chips or ground corncobs. Fresh water may be provided using water bottles or via an automatic watering system. For newly weaned rats, it is advisable to gang house the animals for several days after receipt (e.g., 2–3/cage) to allow the animals to become accustomed to the food and water sources.

This is particularly true if the animals are to be introduced to an automatic water system for the first time.

Each rat should be checked daily to ensure that an adequate supply of fresh food and water is available. In addition, the health of each animal should be verified at the time of receipt and on a daily basis thereafter by trained and qualified personnel. Minimum cage space requirements for the laboratory rat are presented in Table 1.17.

Environmental Conditions

Environmental conditions recommended for rats by the *Guide* are as follows:

Room temperature: 68°F–79°F, 20°C–26°C
 Relative humidity: 30%–70%
 Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges to avoid extremes and possible large fluctuations in these variables. The *Guide* does not specify any particular lighting cycle for rats; however, a 12 h light/12 h dark cycle is routinely used for this species.

Physical and Physiological Parameters

Physical and physiological parameters for laboratory rats are listed in Table 1.18.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Rats (Table 1.19)**Diseases**

Various diseases and adverse health conditions of laboratory rats are identified and briefly described in Table 1.20.

TABLE 1.16
Common Stocks and Strains of Laboratory Rats

Strain	Description
Sprague–Dawley rats	Outbred albino strain originated by R.W. Dawley from a hybrid hooded male and female Wistar rat. This designation includes variants such as the CD®IGS rat. In general, the Sprague–Dawley rat and its variants constitute a hardy multipurpose animal model with a rapid growth rate.
Wistar Han rats	Outbred albino strain originally bred at the Hannover Institute as a distinct line divergent from the strain that originated at the Wistar Institute. This strain is preferred for carcinogenicity studies due to its smaller body size, lower spontaneous tumor incidence, and greater survival.
Long–Evans rats	Outbred white with black or occasional brown hood; originated by Drs. Long and Evans by cross of white Wistar females with wild gray male. A multipurpose model often used in obesity and behavioral research.
Zucker Diabetic Fatty rats	Inbred line derived from outbred obese Zucker rats used primarily for research on diabetes and obesity.
Fischer 344 (F-344) rats	Multipurpose inbred albino strain originated from mating #344 of rats obtained from local breeder (Fischer).
Lewis rats	Inbred albino strain originally developed by Dr. Lewis from Wistar stock. Principal research uses include induced anemia, induced arthritis/inflammation, induced type I diabetes, and transplantation research.
Wistar Kyoto (WKY) rats	Inbred albino strain originated from outbred Wistar stock from Kyoto School of Medicine. Used as a control for the SHR rat and ADHD model.
Spontaneously hypertensive (SHR) rates	Inbred albino strain developed from Wistar Kyoto rats with spontaneous hypertension. Used in hypertension research and as an animal model for ADHD. Variants include the Spontaneously Hypertensive Heart Failure (SHHF) Rat, the Spontaneously Hypertensive Obese (SHROB) Rat, and the Spontaneously Hypertensive Stroke-Prone (SHRSP) Rat.

TABLE 1.17
Minimum Cage Space Requirements for Rats^a

Body Weight (g)	Floor Area/Rat, in. ² (cm ²)	Cage Height, in. (cm)
<100	17.0 (109.6)	7 (17.8)
100–200	23.0 (148.35)	7 (17.8)
200–300	29.0 (187.05)	7 (17.8)
300–400	40.0 (258.0)	7 (17.8)
400–500	60.0 (387.0)	7 (17.8)
>500	>70.0 (≥451.5)	7 (17.8)

^a As per the National Research Council of the National Academies, 2011.

TABLE 1.18
Physical and Physiological Parameters of Rats

Life span	2–3 years
Male adult weight	350–400 g
Female adult weight	180–200 g
Birth weight	5–6 g
Adult food consumption	10–20 g/day
Adult water consumption	20–30 mL/day
Male breeding age/weight	10–12 weeks/300–350 g
Female breeding age/weight	8–10 weeks/200–300 g
Placentation	Discoidal hemochorial
Estrus cycle	4–5 days (polyestrous)
Gestation	20–22 days
Weaning age/weight	21 days/35–45 g
Average litter size	10–12 pups
Mating system(s)	1:1 or 1 male to multiple females
Adult blood volume	6%–7% of body weight
Maximum safe bleed	5–6 mL/kg
Red cell count	6–10 × 10 ⁶ /mm ³
White cell count	7–14 × 10 ³ /mm ³
Hemoglobin	11–18 g/dL
Hematocrit	34%–48%
Mean corpuscular volume	50–65
Mean corpuscular hemoglobin	19–23
Mean corpuscular hemoglobin concentration	32–38
Platelet count	800–1500 × 10 ³ /mm ³
Heart rate	250–500 beats/min
Respiration rate	80–150 breaths/min
Rectal temperature	37.5°C
Urine pH	6.0–7.5
Urine volume	10–15 mL/day
Chromosome number	2n = 42

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Williams, C.S.F., *Practical Guide to Laboratory Animals*, C.V. Mosby, St. Louis, MO, 1976; *LAMA Lines*, Newsletter of the Laboratory Animal Management Association, 4, September/October, 1988.

TABLE 1.19
Identification, Bleeding, Anesthesia, and Euthanasia Methods for Laboratory Rats

Identification methods
Ear tags
Ear punch, notch
Tail tattoo identification number
Subcutaneously implanted transponder
Bleeding methods
Orbital sinus
Tail vein and artery
Anesthesia methods
75 mg/kg ketamine and 5 mg/kg xylazine
4–5 mg/100 g body weight sodium pentobarbital
Euthanasia methods
CO ₂ inhalation
Sodium pentobarbital overdose

TABLE 1.20

Various Diseases and Adverse Health Conditions of Laboratory Rats

Disease/Health Condition	Etiology, Clinical Signs, Symptoms, and/or Pathology
Mycoplasma pulmonis	Bacterial infection which is common in conventionally reared rats and mice; responsible for rhinitis, otitis, laryngitis, tracheitis, bronchiolitis, bronchopneumonia, and additionally perioophoritis and salphingitis. Other mycoplasma species have been isolated from rats but <i>M. pulmonis</i> is the only significant pathogen.
Klebsiellosis	Caused by bacterium <i>Klebsiella pneumoniae</i> ; rats with natural disease may have submaxillary, parotid, or inguinal lymph node abscesses.
Tyzzer's disease	Caused by bacterium <i>Clostridium piliforme</i> ; may occur in mice, rats, gerbils, hamsters, guinea pigs, rabbits, cats, dogs, nonhuman primates, horses, and other species; has been reported in Europe, North America, and Asia; most outbreaks in laboratory rats and mice have occurred in conventional colonies; usually subclinical; signs may include diarrhea, poor coat, and sudden death in young; focal necrosis in liver and inflammation of ileum.
Bordetellosis	Caused by bacterium <i>Bordetella bronchiseptica</i> that is a common inhabitant of the respiratory tract of rats and mice; may cause pneumonia usually in association with a primary pathogen such as mycoplasma.
Pasteurellosis	Caused by bacterium <i>Pasteurella pneumotropica</i> , an opportunistic organism; associated with abortion and respiratory, ear, reproductive, mammary gland, conjunctival, and skin lesions; usually a co-pathogen with respiratory pathogens such as Sendai virus and mycoplasma.
Sendai virus	Caused by <i>Paramyxovirus</i> ; extremely contagious; clinically inapparent chronic infection or clinically apparent acute infection; variable signs may include chattering, mild respiratory distress, prolonged gestation, poor growth, and death in young; concurrent pulmonary infections may occur.
Sialodacryoadenitis (SDA)	Caused by <i>Coronavirus</i> ; highly contagious; one of the most common viruses in laboratory rats; virus is present in tissues of infected rats for only about 7 days; suckling rats may have mild transient signs (e.g., conjunctivitis); alternatively, sudden high prevalence of overt disease may occur with signs such as cervical edema, sneezing, photophobia, nasal and ocular discharge, and corneal lesions; there is usually high morbidity and no mortality; histopathological changes in salivary and lacrimal glands are characteristic.
Rat parvoviruses	Rat Virus (RV), H-1 Virus, and Rat Parvovirus (RPV); all are common, but only RV is associated with natural disease. RV is usually subclinical, but can be associated with fetal resorption, neonatal cerebellar hypoplasia with ataxia, hepatitis, jaundice, steatorrhea, and hemorrhages in adults especially when immunosuppressed.
Pneumonia virus of mice	Caused by <i>Pneumovirus</i> ; common infection in laboratory rodents worldwide; active infection lasts about 9 days; natural infections are subclinical in euthymic rodents.
<i>Pneumocystis carinii</i>	Is the causative agent for interstitial pneumonia in laboratory rats; was previously known by the working name "rat respiratory virus" or "RRV".
Ringtail	Condition of young rats and mice characterized by annular constriction and subsequent edema, necrosis, and sloughing of the tail; much more common in rats; may be prevented by providing relative humidity $\geq 50\%$.

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, *Infectious Diseases of Mice and Rats*, National Academy Press, Washington, DC, 1991; Clarence, M.F., *The Merck Veterinary Manual*, 7th edn., Merck & Co., Rahway, NJ, 1991.

GUINEA PIG (*CAVIA PORCELLUS*)

Breeds and Strains

Compared with the variety of mouse and rat strains which are available commercially, relatively few strains of guinea pigs are produced commercially for research purposes. The most commonly used guinea pig strains are albino outbreeds of the Dunkan–Hartley and Hartley varieties. These strains have short, smooth hair and characteristic nonpigmented eyes.

Hairless guinea pigs such as the IAF strain are available from Charles River Laboratories, Wilmington, MA. Although these guinea pigs are a bit more expensive than the standard Hartley guinea pigs, the absence of hair and intact immune system of the hairless guinea pig makes it an

attractive alternative for dermal studies, such as delayed contact hypersensitivity or photoirritation and photosensitization studies. Consequently, the hairless guinea pig seems to be gaining in popularity among scientists involved in dermatological research.

Handling and Restraint

Although the guinea pig is probably the most nervous and panicky species used in research, it generally will not scratch or bite when picked up, provided it is well supported. It is advisable to approach and handle the guinea pig in a quiet and confident manner to avoid inducing any unnecessary stress in the animal. The guinea pig may be picked up easily by placing one hand firmly around the animal's thorax and the other hand beneath the body to support the

animal's weight. The guinea pig will often wiggle and vocalize (whistle) when handled.

Housing

Because guinea pigs cannot jump or climb very well, they may be housed in relatively shallow cages with solid bottoms or wire flooring. If solid-bottom caging is used, the animals should be provided with some type of bedding material, such as processed hardwood chips. If suspended stainless steel caging is used, the wire floor must not allow the animal's feet to extend between the grids, or the animal may be injured accidentally. Water may be supplied to guinea pigs using water bottles or by an automatic watering system. Each guinea pig should be checked daily to ensure that it has an adequate supply of fresh, uncontaminated food and water. Like humans and nonhuman primates, guinea pigs require regular doses of vitamin C (ascorbic acid) to avoid developing scurvy. Fortunately, commercial diets are available that contain an adequate supplement of vitamin C for this species.

The health of each animal should be verified at the time of receipt and on a daily basis thereafter by trained and qualified personnel. Guinea pigs can be rather messy laboratory animals because they quickly foul their cages and seem to enjoy spilling their food and playing with their water sippers. Therefore, the condition of the cages and bedding should be monitored closely and cleaned regularly to provide a suitable habitat for the animals. Minimum cage space requirements for the guinea pig are provided in Table 1.21.

Environmental Conditions

Environmental conditions for guinea pigs recommended by the *Guide* are as follows:

Room temperature: 68°F–79°F, 20°C–26°C
Relative humidity 30%–70%
Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges listed earlier to avoid extremes and large fluctuations in these environmental variables. The *Guide* does not specify any particular lighting cycle for guinea pigs; however, a 12 h light/12 h dark cycle is routinely used for this species.

Physical and Physiological Parameters

Physical and physiological parameters of guinea pigs are listed in Table 1.22.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Guinea Pigs (Table 1.23)

Diseases

Various diseases and adverse health conditions of guinea pigs are identified and briefly described in Table 1.24.

TABLE 1.21
Minimum Cage Space Requirements for Guinea Pigs^a

Body Weight (g)	Floor Area/Guinea Pig, in. ² (cm ²)	Cage Height, in. (cm)
≤350	60.0 (387.0)	7 (17.8)
>350	>101.0 (≥651.5)	7 (17.8)

^a As per National Research Council of the National Academies, 2011.

TABLE 1.22
Physical and Physiological Parameters of Guinea Pigs

Life span	4–6 years
Male adult weight	1000–1200 g
Female adult weight	850–900 g
Birth weight	90–120 g
Adult food consumption	20–30 g/day
Adult water consumption	12–15 mL/100 g
Dietary peculiarities	Vitamin C required to avoid scurvy
Male breeding age/weight	11–12 weeks/600–700 g
Female breeding age/weight	7–8 weeks/350–450 g
Placentation	Discoidal hemochorial
Estrus cycle	16–18 days
Gestation	65–70 days
Weaning age/weight	7–14 days/150–200 g
Litter size	2–5
Mating	1M:1F or 1M:10F
Adult blood volume	6%–7% body weight
Maximum safe bleed	7–8 mL/kg
Red cell count	4.5–7 × 10 ⁶ /mm ³
White cell count	5–15 × 10 ³ /mm ³
Hemoglobin	11–17 g/dL
Hematocrit	39%–47%
Platelet count	250–750 × 10 ³ /mm ³
Heart rate	230–300 beats/min
Respiration rate	60–110 breaths/min
Rectal temperature	39.5°C
Urine pH	8.0–9.0
Urine volume	15–75 mL/day
Chromosome number	2n = 64

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Williams, C.S.F., *Practical Guide to Laboratory Animals*, C.V. Mosby, St. Louis, MO, 1976; *LAMA Lines*, Newsletter of the Laboratory Animal Management Association, 4, September/October, 1988.

TABLE 1.23
Identification, Bleeding, Anesthesia, and Euthanasia Methods
for Laboratory Guinea Pigs

Identification methods
Ear tags
Subcutaneously implanted transponder
Bleeding methods
Cardiac puncture (only with prior anesthesia)
Anesthesia methods
Ketamine, methoxyflurane, isoflurane, pentobarbital
Euthanasia methods
CO ₂ inhalation
Sodium pentobarbital overdose

TABLE 1.24
Various Diseases and Adverse Health Conditions of Guinea Pigs

Disease/Health Condition	Etiology, Clinical Signs, Symptoms, and/or Pathology
Antibiotic-induced toxicity	Guinea pigs and hamsters are highly susceptible to the toxic effects of many antibiotics; toxicity results from overgrowth of <i>Clostridium difficile</i> and subsequent elaboration of toxins; enterocolitis with diarrhea and death may occur in 3–7 days.
Conjunctivitis	Often caused by <i>Chlamydia psittaci</i> ; <i>Salmonella</i> spp., <i>Streptococcus</i> spp., <i>Staphylococcus</i> spp., and <i>Pasteurella multocida</i> may also be involved; clinical signs include conjunctival hyperemia and chemosis with purulent ocular exudate; may be treated with ophthalmic antibiotics.
Lymphadenitis (lumps)	Inflammation and enlargement of the cervical lymph nodes is common in guinea pigs; usually caused by <i>Streptococcus zooepidemicus</i> ; clinical findings are large unilateral or bilateral swellings or abscesses in the ventral neck region; organisms may gain entry to lymphatics from abrasions of the oral mucosa; thus, abrasive materials in feed or litter should be avoided; affected animals should be culled.
Metastatic calcification	Occurs most often in male guinea pigs >1 year old, but is usually clinically inapparent; signs include stiff joints and high mortality; calcium deposits may be seen in the lungs, liver, heart, aorta, stomach, colon, kidneys, joints, and skeletal muscles at necropsy; may be due to diets low in magnesium and potassium.
Muscular dystrophy	Guinea pigs are extremely sensitive to vitamin E deficiency; clinical signs are stiffness, lameness, and refusal to move; microscopic lesions include coagulative necrosis, inflammation, and proliferation of skeletal muscle fibers.
Parasitic diseases	Several protozoa (<i>Toxoplasma gondii</i> , <i>Eimeria caviae</i> , <i>Encephalitozoon cuniculi</i>), nematodes (<i>Paraspidodera uncinata</i>), and lice (<i>Gyropus ovalis</i> , <i>Gliricola porcelli</i>) may infect guinea pigs.
Pneumonia	May be caused in guinea pigs by several bacteria (e.g., <i>Bordetella bronchiseptica</i> , <i>Streptococcus zooepidemicus</i> , <i>S. pneumoniae</i> , <i>Klebsiella pneumoniae</i> , and <i>Pasteurella pneumotropica</i>); clinical signs are respiratory distress; affected animals should be culled.
Pregnancy toxemia	Metabolic disorder caused by obesity and stress which might induce temporary anorexia during late pregnancy; clinical findings are anorexia, adiposia, muscle spasms, coma within 48 h of onset, and death within 4–5 days; laboratory findings are aciduria, proteinuria, hyperlipemia, and fatty degeneration of parenchymatous organs.
Ringworm	Dermatophytic infection usually caused in guinea pigs by <i>Trichophyton mentagrophytes</i> ; signs are alopecia (usually starting at head), characterized by crusty, flaking lesions of the skin; facial lesions are common and disease may spread over the posterior regions; contagious to man and other animals.
Scurvy	Caused by vitamin C deficiency; guinea pigs cannot synthesize or appreciably store vitamin C; clinical signs include unsteady gait, painful locomotion, hemorrhage of gums, swelling of joints, and emaciation; may be prevented by providing 15–25 mg of vitamin C per day; vitamin C is stable for 3 months in commercial guinea pig diets that are properly stored after milling.
Slobbers	Actually represents several conditions characterized by wet, matted hair around the mouth, chin, and ventral neck; drooling occurs whenever mastication is impaired (e.g., from dental abnormalities such as malocclusion or mandibular deformity); incisor teeth may be clipped to improve occlusion; mandibular deformity may result from subacute scurvy, folate deficiency, or excess dietary fluoride.

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Clarence, M.F., *The Merck Veterinary Manual*, 7th edn., Merck & Co., Rahway, NJ, 1991.

RABBIT (*ORYCTOLAGUS CUNICULUS*)

Breeds and Strains

There are more than 100 different breeds and varieties of rabbits recognized by the American Rabbit Breeders Association. However, relatively few breeds are used for research purposes. Two breeds which have been used quite extensively for research include the New Zealand White rabbit and the Dutch Belted rabbit.

The New Zealand White (NZW) rabbit is an albino breed with rather large ears and characteristic nonpigmented eyes. This breed commonly attains a mature body weight of approximately 4.5 kg (approximately 10 lb). In contrast, the Dutch Belted rabbit is a nonalbino breed that is usually black with a white stripe on the face and around the thorax. This breed has a mature body weight which is substantially less than that of the NZW rabbit, in the range of 2–2.5 kg (approximately 4.5–5 lb).

Handling and Restraint

As with all species, it is essential that personnel receive proper, supervised training in appropriate handling and restraint techniques before working with rabbits. The rabbit should **NEVER BE PICKED UP BY THE EARS ALONE**, because this will undoubtedly result in injury to the animal, and possibly to the caretaker. If handled incorrectly, the rabbit usually becomes excited and kicks viciously with its powerful back legs while twisting and contorting its body. This often leads to spinal injury to the animal.

The best method for picking up the rabbit is to grasp the scruff of the neck with one hand, while supporting the rump of the animal with the other hand. A gentle but firm grip is necessary to control the animal and to reduce anxiety and fear which are associated with restraint and lifting of the animal. Because all rabbits resist restraint to some degree, handlers should wear long sleeves or other protective covering to avoid being scratched.

Housing

In the laboratory setting, rabbits are usually housed in individual, suspended stainless steel cages with wire or slatted metal flooring. Fresh water may be supplied by water bottles or via an automatic watering system. Commercial rabbit feed is usually supplied using “J-type” feeders in which the curved lower portion dwells inside the cage and the square “hopper” portion is attached to the outside of the cage. Because male rabbits commonly express urine outside their cages, it is desirable to use some type of feeder lid to avoid contamination of the food supply of animals on lower cage levels. Food may be provided *ad libitum*, or on a restricted daily basis (e.g., 30–60 g/kg/day for an adult rabbit). When fed *ad libitum*, rabbits (like some humans) often gorge themselves and eat more than is really needed. A large NZW rabbit can usually maintain its body weight while being fed no more than 110–150 g of pellets per day.

Rabbits produce two types of feces that are known as “day feces” and “night feces.” The day feces are hard and round, whereas the night feces are soft and covered by gray mucus. The night feces are consumed by the rabbit directly from the anus in a practice known as “coprophagy.” This provides the rabbit with some benefit in the way of increased digestibility of protein and vitamins. Because of this practice, overnight fasting of rabbits will rarely, if ever, result in complete emptying of the stomach. Use of cages with wire flooring does not in any way reduce or eliminate coprophagy.

As with all species, the health of each rabbit should be verified at the time of receipt and on a daily basis thereafter by trained and qualified personnel. The condition of the animals’ cages should be closely monitored and cleaned regularly to provide a suitable habitat for the animals. Minimum cage space requirements for rabbits are presented in Table 1.25.

Environmental Conditions

Environmental conditions recommended for rabbits by the *Guide* are as follows:

Room temperature: 61°F–72°F, 16°C–22°C
Relative humidity: 30%–70%
Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges just described to avoid extremes and large fluctuations in these environmental variables. The *Guide* does not specify any particular lighting cycle for rabbits; however, a 12 h light/12 h dark cycle is routinely used for this species.

Physical and Physiological Parameters

Physical and physiological parameters for laboratory rabbits are listed in Table 1.26.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Rabbits (Table 1.27)

Diseases

Various diseases and adverse health conditions of rabbits are identified and briefly described in Table 1.28.

TABLE 1.25
Minimum Cage Space Requirements
for Rabbits^a

Body Weight (kg)	Floor Area/ Rabbit ft ² (m ²)	Cage Height in. (cm)
<2.0	1.5 (0.14)	16 (40.5)
2–4.0	3.0 (0.28)	16 (40.5)
4–5.4	4.0 (0.37)	16 (40.5)
>5.4	≥5.0 (≥0.46)	16 (40.5)

^a As per the National Research Council of the National Academies, 2011.

TABLE 1.26
Physical and Physiological Parameters of Rabbits

Life span	5–7 years
Male adult weight	4.0–5.5 kg
Female adult weight	4.5–5.5 kg
Birth weight	90–110 g
Adult food consumption	75–100 g
Adult water consumption	80–100 mL/kg body weight
Dietary peculiarities	Pelleted diet
Male breeding age/weight	6–7 months/3.5–4.0 kg
Female breeding age/weight	5–6 months/4.0–4.5 kg
Placentation	Discoidal hemoendothelial
Estrus cycle	Polyestrous, induced
Gestation	30–32 days
Weaning age/weight	6–7 weeks/1.0–1.5 kg
Litter size	4–12
Mating system(s)	1:1 or via artificial insemination
Adult blood volume	6% of body weight
Maximum safe bleed	6.5–7.5 mL/kg
Red cell count	4.5–7.0 $\times 10^6/\text{mm}^3$
White cell count	5–12 $\times 10^3/\text{mm}^3$
Hemoglobin	11–14 g/dL
Hematocrit	32%–48%
Mean corpuscular volume	58–72
Mean corpuscular hemoglobin	18–24
Mean corpuscular hemoglobin concentration	30–35
Platelet count	250–750 $\times 10^3/\text{mm}^3$
Heart rate	250–300 beats/min
Respiration rate	35–55 breaths/min
Rectal temperature	39.5°C
Urine pH	8.2
Urine volume	50–130 mL/kg
Chromosome number	2n = 44

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Williams, C.S.F., *Practical Guide to Laboratory Animals*, C.V. Mosby, St. Louis, MO, 1976; *LAMA Lines*, Newsletter of the Laboratory Animal Management Association, 4, September/October, 1988.

TABLE 1.27
Identification, Bleeding, Anesthesia, and Euthanasia Methods for Laboratory Rabbits

Identification methods
Ear studs/tags
Ear tattoo
Subcutaneously implanted transponder
Bleeding methods
Marginal ear vein
Jugular vein
Cardiac puncture (only with prior anesthesia)
Anesthesia methods
Halothane, pentobarbital, methoxyflurane, ketamine, xylazine
Euthanasia methods
Barbiturate overdose (IV)
Other chemical euthanasia solutions

TABLE 1.28
Various Diseases and Adverse Health Conditions of Rabbits

Disease/Health Condition	Etiology, Clinical Signs, Symptoms and/or Pathology
Pasteurellosis	Common and highly contagious disease caused by <i>Pasteurella multocida</i> ; may be transmitted by direct or indirect contact; some animals may be asymptomatic carriers; infection may manifest as rhinitis (snuffles), pneumonia, otitis media, conjunctivitis, abscesses, genital infections, or septicemia; best controlled by strict culling; rhinitis, abscesses, genital infection, and pneumonia due to <i>P. multocida</i> and other agents are described further in the table.
Rhinitis	<i>Pasteurella</i> —induced acute, subacute, or chronic inflammation of the air passages and lungs; serous exudate from nose and eyes may become purulent; fur on inside of front legs may become matted due to pawing at nose; infected animals usually sneeze and cough; infection may proceed to pneumonia.
Abscesses	<i>Pasteurella</i> -induced abscesses may be found in any part of the body or head; fight wounds may develop into abscesses; it is usually better to eliminate rather than treat affected rabbits.
Genital infections	Genital infections may be caused by <i>Pasteurella</i> or other organisms; the infections manifest as acute or subacute inflammation of the reproductive tract; occurs more often in does than bucks; females may exhibit yellowish gray vaginal discharge; best controlled by culling.
Pneumonia	<i>P. multocida</i> accounts for most cases, but other bacteria may be involved (e.g., <i>Klebsiella pneumoniae</i> , <i>Bordetella bronchiseptica</i> , and <i>pneumococci</i>); upper respiratory disease (snuffles) often precedes pneumonia; occurrence may be directly proportional to level of ammonia in rabbitry; affected animals usually die within 1 week after signs appear.
Listeriosis	Sporadic septicemic disease caused by <i>Listeria monocytogenes</i> and characterized by sudden deaths, abortions, or both; clinical signs are nonspecific and may include anorexia, depression, and weight loss; seldom affects the CNS but may spread to the liver, spleen, and gravid uterus; multiple gray-white foci are commonly seen at necropsy.
Staphylococcosis	Caused by <i>Staphylococcus aureus</i> ; manifests as fatal septicemia in young rabbits and suppurative inflammation in older rabbits involving almost any organ or tissue; infected rabbits may show no signs until resistance is decreased; abscesses develop in chronic infections; usually fever, depression, anorexia, and then death in acute septicemia.
Enterotoxemia	Explosive diarrheal disease of young rabbits (e.g., 4–8 weeks of age) which occasionally affects adults; one recognized cause is <i>Clostridium spiroforme</i> ; signs are lethargy, rough coat, greenish brown fecal staining, and death within 48 h; necropsy shows fluid-distended intestine with petechiae on serosal surface.
Mucoid enteropathy	Diarrheal disease which may occur in rabbits of any age; exact etiology is unknown; clinical signs are mucoid feces, anorexia, lethargy, dehydration, rough coat, bloated abdomen, and perineal area covered with mucus and feces; impaction of cecum and gelatinous mucus in the colon are common necropsy findings.
Tyzzer's disease	Caused by <i>Bacillus piliformis</i> ; produces severe diarrhea and death in young rabbits; characterized by profuse diarrhea, anorexia, dehydration, lethargy, and death in 1–3 days; bacterium may affect other species.
Hepatic coccidiosis	Caused by <i>Eimeria stiedae</i> ; transmission is by ingestion of sporulated oocysts; severity of disease depends on the number of oocysts ingested; rabbits may fail to make normal gains, but infection is usually asymptomatic; small yellowish white nodules are found throughout the hepatic parenchyma at necropsy; microscopically, nodules are composed of hypertrophied bile ducts; oocysts may be demonstrated by fecal flotation and microscopic examination.
Intestinal coccidiosis	Caused by <i>E. magna</i> , <i>E. irresidua</i> , <i>E. media</i> , <i>E. perforans</i> , or other <i>Eimeria</i> spp.; in hepatic coccidiosis, transmission is by ingestion of sporulated oocysts; may occur in rabbits receiving the best of care; infections are usually mild with no clinical signs; intestines may become thickened and pale; oocysts may be demonstrated by fecal flotation and microscopic examination.
Ear mites	The ear mite, <i>Psoroptes cuniculi</i> , is a common parasite of rabbits; common signs are head shaking, ear flopping, and ear scratching; ears can be treated by removing exudate and applying miticide for dogs and cats, or light mineral oil alone; treatment should be repeated in 6–10 days and continued as necessary; ivermectin has been shown effective in treating ear mites.
Fur mites	Infestation is usually asymptomatic unless animals become debilitated; infestations are common by <i>Cheyletiella parasitovorax</i> and <i>Leporacarus (Listrophorus) gibbus</i> ; occasional small scabs and sores may be seen on the necks of adult animals.
Encephalitozoonosis	Widespread protozoal infection of rabbits caused by <i>Encephalitozoon (Nosema) cuniculi</i> ; occasionally infects mice, guinea pigs, rats, and dogs; usually no clinical signs are seen; pitting of the kidneys may be seen at necropsy; microscopic lesions consist of focal granulomas and pseudocysts in the brain and kidneys.
Pinworms	The rabbit pinworm, <i>Passalurus ambiguus</i> , is usually not clinically significant; adult worm lives in cecum or anterior colon; not transmissible to man.
Pox viruses	Caused by Rabbitpox Virus which is closely related to vaccinia virus and the same as rabbit plaque or “pockless” rabbitpox; transmission is via direct contact (nasal secretions); clinical signs include fever, nasal and ocular discharge, enlarged lymph nodes, and typical pox lesions in the skin with associated high mortality; lesions include papules or nodules in the dermis (central necrosis with mononuclear cell infiltration), possible necrosis and hemorrhages in lung, spleen, lymph nodes, liver, testis, ovary, and uterus.
Orthopox	

(continued)

TABLE 1.28 (continued)
Various Diseases and Adverse Health Conditions of Rabbits

Disease/Health Condition	Etiology, Clinical Signs, Symptoms and/or Pathology
Leporipox	Rabbit Myxoma Virus is worldwide; transmission is mechanical via direct contact or arthropod vectors; rare in laboratory rabbits, endemic in the wild with 99% mortality in susceptible <i>Oryctolagus</i> spp.; lesions vary markedly due to virulence and resistance factors; clinical signs include dermal masses, gleatinous edema, especially around body orifices and face; lesions in the skin display proliferation of “myxoma cells” (undifferentiated stellate mesenchymal cells) in dermis with abundant mucinous matrix; epidermis may be hyperplastic to degenerate with numerous eosinophilic intracytoplasmic inclusions; hemorrhage or necrosis as well as myxoma cells may be seen in other organs. Rabbit Fibroma Virus (Shope Fibroma Virus) occurs under natural conditions in cottontail rabbit; has been experimentally induced by <i>Oryctolagus</i> spp.; transmission is mechanical via arthropods; infection may be limited to subcutaneous rubbery masses that develop 3–5 days post exposure (PE); these masses display mesenchymal/fibroblastic proliferation in the superficial dermis with epithelial hyperplasia extending into the mass; at 10–15 days PE regression can be seen with infiltration of lymphocytes and plasma cells, and necrosis; lesions associated with a subtype metastatic virus consist of subcutaneous fibromas at 3–6 days PE with systemic metastases, and reduced T and B-cell response in the spleen, death by PE day 10–14 from bronchopneumonia.
Papova Viruses (Papillomatosis)	Cottontail Rabbit Papilloma Virus (CRPV) is also called Rabbit or Shope Papilloma Virus; natural infection in wild rabbits, rare in domestic rabbits; infects skin, never the oral cavity; spread by direct contact and insects; lesions include long keratinized papillary projections (warts) that may persist for months; this lesion may progress to squamous cell carcinoma. Oral Papillomatosis Virus (OPV) infects nonkeratinized surfaces only; usually ventral aspects of tongue or, rarely, ventral oral cavity; never elsewhere on the body; growths are typically solitary and papillary that regress in weeks to 1–2 years; the CRPV and OPV are distinctly different viruses and do not cross-react.
Viral hemorrhagic disease	Highly contagious acute infection primarily of domestic rabbits; causative agent is a calicivirus; lactating and gestating females are most susceptible; rabbits are often found dead with no prior signs; clinical signs in protracted cases include dyspnea, congestion of eyelids, abdominal respiration, and tachycardia; death may be preceded by violent cage activity such as rapid turns and flips; bloody nasal discharge is sometimes seen; gross lesions are generally limited to congestion of the respiratory tract and liver; microscopically there may be marked focal coagulative hepatic necrosis; focal necrosis of the myocardium may also be seen.
Broken back	Fracture or dislocation of lumbar vertebrae is common; signs include posterior paresis or paralysis and urinary and fecal incontinence; this condition warrants immediate euthanasia
Cannibalism	Young does may kill and consume their young for any number of reasons; cannibalism of dead young is a natural nest-cleaning activity.
Dental malocclusion	Overgrowth of incisors can result in difficulty in eating and drinking; this can be corrected by cutting the teeth from time to time.
Hair chewing and hairballs	Hair may accumulate in the stomach due to grooming; excessive hair may produce blockage resulting in anorexia, weight loss, and death.
Ulcerative pododermatitis (sore hocks)	Commonly caused by body-weight pressure on wire-floored cages; factors such as accumulation of urine-soaked feces and the type of wire flooring can influence development; there is no effective treatment; affected animals should be culled.

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Clarence, M.F., *The Merck Veterinary Manual*, 7th edn., Merck & Co., Rahway, NJ, 1991.

DOG (*CANIS FAMILARIS*)

Breeds and Strains

A variety of breeds and strains of dogs are used for laboratory studies. For toxicology investigations, the pure-bred beagle is probably the most popular breed owing to its uniform and relatively small size, docile temperament, physiological similarities to humans, and ability to adapt well to cage life. Beagles and other pure-bred strains produced specifically for research purposes are available from a number of USDA-licensed commercial suppliers (see Table 1.9). These “bred for research” dogs offer the additional advantages of known genetic pedigrees and documented health histories and therapeutic treatments.

Receipt

As with all species, the health of each dog should be verified at the time of receipt and on a daily basis thereafter by trained and qualified personnel. On receipt, each dog should be given a thorough physical examination by a qualified veterinarian. The extent of this examination may vary, but, as a minimum, it should include general physical appearance and behavior, assessment of heart and lung sounds, presence of external parasites, condition of gums and teeth, mobility, and presence of any abnormal secretions or signs of gastrointestinal disturbance. Additional evaluations should be undertaken as deemed appropriate by the veterinarian or required by laboratory standard operating procedures. These may include fecal flotation for internal parasites, hematology and clinical

chemistry assessments, ophthalmological examinations, and ECG measurements.

Each dog must have a certificate of health that has been completed by a licensed veterinarian and states that the animal was determined to be in acceptable condition no longer than 10 days before shipping. The date of last access to feed and water must appear on the paperwork accompanying each dog and on the dog's transport cage. The paperwork must also provide a place to document that feed was offered at least every 24 h for dogs 16 weeks of age or older, and at least every 12 h for dogs less than 16 weeks of age. Potable water must be offered at least once every 12 h to all dogs in transit.

Handling and Restraint

Compassionate treatment and commonsense are the cornerstones of proper management of dogs and other laboratory species. Specifications for the humane handling, care, treatment, and transportation of dogs (and cats) may be found in Part 3, Subpart A of the Animal Welfare Act.¹ Other regulations concerning USDA licensing, registration, research facilities, veterinary care, animal identification, required records, and regulatory compliance may be found in Part 2, Subpart A of the Animal Welfare Act.

The dog may be handled and restrained in a safe manner, provided that personnel are properly trained and experienced in appropriate handling and restraint techniques. Smaller dogs such as the beagle may be lifted by extending one arm under the abdominal area in front of the hind legs and the other arm in front of the chest area. Like other species, dogs should be handled in a firm but gentle manner.

During experimental manipulation (e.g., dose administration, clinical and physical examinations, blood sampling, electrocardiograms, etc.), it is wise to employ a team of two individuals, one to provide restraint and reassurance to the dog while the other performs experimental procedures. For some experimental techniques, the dog may be placed in a suspended upright position using a body sling designed for this purpose. Alternatively, the dog may be placed and manually restrained on top of an examination table. Irrespective of the method used, the presence of a second individual is most helpful to provide reassurance to the animal during restraint.

Occasionally, even docile breeds such as the beagle may become aggressive and require muzzling and/or chemical restraint to protect laboratory personnel. Muzzles may be purchased for this purpose, or simply prepared by wrapping the animal's snout with a gauze. When aggressive animals are encountered, the investigator and facility veterinarian should attempt to determine the reason for the adverse behavior and take immediate action. If the animal is injured, it should be euthanized. If the aggressive behavior is a manifestation of experimental treatment, immediate steps should be taken to ensure the safety of laboratory personnel. Of course, these latter comments apply to all species used in laboratory research.

Housing

Although the dog is hardier than many laboratory species, it must still be provided with a safe and comfortable habitat which

protects the animal from extreme temperatures and weather conditions that may be uncomfortable or hazardous to the animal.

Dogs may be housed in cages, pens, or runs, with one or more dogs per primary enclosure. However, if housed in group pens or runs, only compatible animals should be placed together. Primary enclosures must be designed and constructed in a suitable and structurally sound manner. The enclosures must be kept in good repair with surfaces that can be readily cleaned and sanitized. Floors must protect the animals' feet and legs from injury. If mesh or slatted floors are used, the dog's feet must not be allowed to pass through the floor openings. Primary enclosures must be cleaned daily and sanitized a minimum of once every 2 weeks. Animals are not to be wetted or contaminated by water from other cages during the cleaning process.

All dogs must have easy and convenient access to fresh food and water. Food must be provided at least once each day, except as otherwise might be required to provide adequate veterinary care. The food must be uncontaminated, wholesome, palatable, and of sufficient quantity and nutritive value to maintain the normal condition and weight of the animal. If potable water is not continually available to the animal, it must be offered as often as necessary to ensure the health and well-being of the dog (i.e., not less than twice daily for at least 1 h each time). Minimum cage space requirements for the dog are presented in Table 1.29.

According to the *Guide*, some dogs, especially those toward the upper limit of each weight range, may require additional floor space or cage height to ensure compliance with the AWA. These regulations mandate that the height of each cage be sufficient to allow the dog to stand in a "comfortable position" and that the minimum square footage of floor space be equal to the "mathematical square of the sum of the length of the dog in inches, as measured from the tip of the nose to the base of the tail, plus 6 inches, expressed in square feet." As an example, the floor space calculation for a dog measuring 28 in. from the tip of the nose to the base of the tail would be

$$\frac{(28 \text{ in.} + 6 \text{ in.})^2}{144 \text{ in.}^2/\text{ft}^2} = 8.0 \text{ ft}^2$$

Environmental Conditions

Environmental conditions recommended for dogs by the *Guide* are as follows:

Room temperature: 64°F–84°F, 18°C–29°C
Relative humidity: 30%–70%
Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges stated earlier to avoid extremes and large fluctuations in these environmental variables. The *Guide* does not specify any particular lighting cycle for dogs; however, a 12 h light/12 h dark cycle is routinely used for this species.

Exercise

Dogs 12 weeks old or older, which are housed separately from other dogs must be given an opportunity to exercise regularly if their cage size is less than two times the required size. Dogs which are gang-housed in a pen that meets 100% of the required floor space (i.e., the sum of each dog's requirement) are not required to be provided with additional exercise. The specific exercise program must be approved by the attending veterinarian and IACUC. The program must be in the written form of a standard operating procedure, and records documenting the regimen must be maintained for each dog. Each dog that is denied contact with another dog must have positive daily physical contact with an animal handler.

Exercise exemptions for health reasons deemed necessary by the veterinarian must be in written form and, unless the health condition is permanent, must be reviewed by the veterinarian every 30 days. The IACUC must also agree to the proposed exercise exemption and must review the proposal at least once each year. If the principle investigator determines that the exercise is detrimental to the research protocol for scientific reasons, exercise may be eliminated, provided that the IACUC approves of this action. In this case, the committee's approval must be in the IACUC-approved protocol.

Physical and Physiological Parameters

Physical and physiological parameters for dogs are listed in Table 1.30.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Dogs (Table 1.31)

Diseases

Various diseases and adverse health conditions of dogs are identified and briefly described in Table 1.32.

TABLE 1.29
Minimum Cage Space Requirements for Dogs^a

Body Weight (kg) ^a	Floor Area/ Dog ^b ft ² (m ²)	Cage Height ^a in. (cm)
<15	8.0 (0.74)	Not applicable
15–30	12.0 (1.2)	Not applicable
>30	≥24.0 (≥2.4)	Not applicable

^a As per the National Research Council of the National Academies, 2011.

^b These guidelines may require modification based on the body conformation of individual animal and breed.

TABLE 1.30
Physical and Physiological Parameters of Dogs

Life span	12–14 years
Male adult weight	6–25 kg
Female adult weight	6–25 kg
Birth weight	300–500 g
Adult food consumption	250–1200 g/day
Adult water consumption	100–400 mL/day
Breeding age (males)	9–12 months
Breeding age (females)	10–12 months
Estrus cycle	Biannual, monestrus
Gestation	56–58 days
Weaning age	6–8 weeks
Litter size	4–8
Mating	Pairs, 1 male to multiple females
Adult blood volume	8%–9%, 75–110 mL/kg
Maximum safe bleed	8–10 mL/kg
Red cell count	5.5–8.5 × 10 ⁶ /mm ³
White cell count	6–14 × 10 ³ /mm ³
Hemoglobin	13–18 g/dL
Hematocrit	38%–52%
Platelet count	200–600 × 10 ³ /mm ³
Heart rate	80–140 beats/min
Respiration rate	10–30 breaths/min
Rectal temperature	38.5°C
Urine pH	7.0–7.8
Urine volume	25–45 mL/kg
Chromosome number	2n = 78

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; *LAMA Lines*, Newsletter of the Laboratory Animal Management Association, 4, September/October, 1988.

TABLE 1.31
Identification, Bleeding, Anesthesia, and Euthanasia Methods for Laboratory Dogs

Identification methods
Chain collar with metal (numbered) tag
Letter tattoo on ear or flank
Cage card + individual animal records
Subcutaneously implanted transponder
Bleeding methods
Cephalic, saphenous, femoral, and jugular veins
Anesthesia methods
Halothane, isoflurane, tranquilizers, narcotics, sodium pentobarbital
Euthanasia methods
Overdose of anesthetic drugs or chemical euthanasia solutions (e.g., sodium pentobarbital)

TABLE 1.32
Various Diseases and Adverse Health Conditions of Dogs

Disease/Health Condition	Etiology, Clinical Signs, Symptoms and/or Pathology
Distemper (hardpad disease)	Caused by a paramyxovirus; highly contagious systemic disease characterized by diphasic fever, leukopenia, gastrointestinal and respiratory catarrh, and frequent pneumonic and neurological complications; suspected cause of multiple sclerosis in man; hyperkeratosis of footpads may occur; CNS signs include localized twitching, paresis, or paralysis, and convulsions with salivation and chewing movements; seizures may become more frequent and severe; atrophy of thymus is a consistent postmortem finding; prevention is available via vaccination.
Parvovirus	An enteritis of acute onset and varying morbidity and mortality; dogs of all ages may be affected, but puppies seem to be more susceptible; older and immune-impaired dogs may also be more susceptible to the virus; virus produces two different disease forms (myocarditis and enteritis); intestinal crypts become infected resulting in collapse of villi and necrosis of crypt cells; clinical signs include anorexia, lethargy, and rapid dehydration; lymphopenia (but not leukopenia) is found in most affected dogs; death may follow due to dehydration, electrolyte imbalance, endotoxic shock, or secondary septicemia; small intestine is primarily affected in enteric form of disease; pulmonary edema is main finding in myocardial form; prevention is available via vaccination.
Hepatitis virus	Contagious disease with signs that vary from slight fever to severe depression, marked leukopenia, prolonged bleeding time, and death; caused by canine adenovirus-1; clinical signs include apathy, anorexia, thirst, conjunctivitis, ocular discharge, nasal discharge, and occasional abdominal pain; disseminated intravascular coagulation is common; liver, kidneys, spleen, and lungs are the main target organs; hepatic cell necrosis and "paint brush" hemorrhages of the gastrointestinal tract, lymph nodes, thymus, and pancreas are observed postmortem; 25% of recovered dogs develop bilateral corneal opacity; prevention is available via vaccination which is often given with distemper immunizations.
Canine herpesvirus	Fatal viral infection of puppies worldwide; transmission occurs between susceptible puppies and the infected dam; death usually occurs at 1–3 weeks; characteristic lesions consist of disseminated focal necrosis and hemorrhages; no vaccine is available, however, subsequent litters receive maternal antibodies in the colostrum and disease does not develop.
Canine coronavirus	Highly contagious gastrointestinal disease of dogs characterized by emesis and diarrhea; signs are similar to parvovirus but usually milder; prevention is available via vaccination.
Parainfluenza virus	Virus is capable of causing disease by itself, but is probably more often involved as a primary infection followed by secondary invaders; clinical signs include fever, anorexia, serous nasal discharge, lacrimation, and coughing; histological lesions include bronchiolitis and alveolitis with marked congestion and hemorrhage; prevention is available via vaccination.
Brucellosis	Caused by <i>Brucella canis</i> ; disease disseminates rapidly among dogs closely kenneled; both sexes seem to be equally susceptible; primary signs are abortion, stillbirths, and conception failures; infected dogs develop generalized lymphadenitis, epididymitis, periorchitis, and prostatitis; transmission is congenital, venereal, or by ingestion of contaminated materials; bacteremia may persist for up to 2 years; attempts at immunization have not been uniformly successful.
Infectious tracheobronchitis (kennel cough)	Highly contagious but generally a mild and self-limiting disease that affects dogs of all ages and results from inflammation of the upper airways; may progress to fatal bronchopneumonia in puppies or chronic bronchitis in adult dogs; spreads rapidly among susceptible dogs; canine parainfluenza virus, canine distemper virus, canine adenovirus-2, or <i>Bordetella bronchiseptica</i> may act as primary pathogens; concurrent infections with several pathogens is common; prominent clinical sign is harsh dry cough which may be followed by retching and gagging; development of more severe signs indicates complicating systemic infection such as distemper or bronchopneumonia; dogs should be immunized against distemper, parainfluenza, and canine adenovirus-2.
Coccidiosis	Approximately 22 species of coccidia infect the intestinal tract of dogs; the most common coccidia of dogs are <i>Cystoisospora</i> spp.; common clinical signs in severe cases are diarrhea (sometimes bloody), weight loss, and dehydration; may be treated with coccidiostatic agents such as sulfadimethoxine or nitrofurazone.
Giardiasis	Intestinal protozoan which causes acute enteritis; trophozoites of <i>Giardia canis</i> may be detected by direct saline smears of fecal samples; ova may be detected by fecal flotation.
Babesiosis	Caused by a protozoan, <i>B. canis</i> , transmitted by a variety of tick genuses. The organism parasitizes red blood cells leading to anemia, fever, lethargy, and poor appetite; in more severe cases, severe depression, drooling, vomiting, jaundice, hemoglobinuria (due to intravascular hemolysis), mucosal petechiae and congestion, ulcerative stomatitis, and angioneurotic edema of the head, legs, and body occur; disseminated intravascular coagulation is a consistent occurrence in severe <i>B. canis</i> ; <i>B. gibsoni</i> is another agent of canine babesiosis, but is apparently restricted to Asia; extravascular hemolysis is the rule with <i>B. gibsoni</i> in which splenomegaly and death due to anemia are common.
Mange	Contagious skin disease caused by several species of mites; may be transmitted by larvae, nymphs, and fertilized females; signs include alopecia and pruritis with intense irritation; if untreated, infestation can lead to emaciation, debilitation, and even death; sarcoptic mites burrow in the skin and cause intense itching, scratching, chewing, and rubbing; this often leads to inflammation and secondary infections; skin becomes dry, thickened, wrinkled, and crusty; demodectic mites feed on cells of the hair follicles and are more likely to produce localized lesions.

(continued)

TABLE 1.32 (continued)
Various Diseases and Adverse Health Conditions of Dogs

Disease/Health Condition	Etiology, Clinical Signs, Symptoms and/or Pathology
Heartworm	Clinical or subclinical disease complex caused by the filarial worm <i>Dirofilaria immitis</i> ; occurs frequently in mosquito-infected areas; duration and severity of infection determine the severity of clinical signs; common findings are coughing, decreased exercise tolerance, and weight loss; large numbers of worms in right atrium and vena cava can cause death; treatment is difficult; affected dogs are not suitable for research.
Roundworm	Roundworms are relatively common in dogs, especially puppies; <i>Toxocara canis</i> is the most important species, because it is fatal in young pups and larvae may migrate in man; infected dogs fail to grow and exhibit dull coats and distended abdomens; signs in severe infestations include pneumonia, ascites, fatty degeneration of liver, and mucoid enteritis; worms may be vomited or voided in feces; animals may be treated with piperazine salts or broad-spectrum compounds such as dichlorvos, febantel, or ivermectin.
Strongyloidosis	Small slender nematode (<i>Strongyloides stercoralis</i>) parasite that inhabits the small intestine of dogs; worms are practically transparent; infections are usually associated with warm, wet, crowded, unsanitary housing; presence of clinical signs indicates heavy infection; these include blood-streaked mucoid diarrhea, emaciation, and reduced growth; in advanced stages, there is shallow rapid breathing and pyrexia (indicates grave prognosis); common postmortem findings are pneumonia with large areas of consolidation, marked enteritis with hemorrhage, mucosal exfoliation, and profuse mucus secretion.
Stomach worm	Stomach nematodes of dogs (<i>Physaloptera</i> spp.) occur throughout the world; these parasites cause gastritis and duodenitis resulting in vomiting, anorexia, and dark feces; anemia and weight loss may develop in heavy infections; treatment with pyrantel and carbon disulfide is effective.
Hookworms	<i>Ancylostoma caninum</i> is the principal cause of canine hookworm disease; a characteristic change in young pups is acute normocytic, normochromic anemia followed by hypochromic, microcytic iron deficiency anemia; this is often fatal; anemia results from blood sucking and ulceration when <i>A. caninum</i> shift feeding sites; hydremia, emaciation, and weakness develop in chronic disease.
Whipworm	The whipworm (<i>Trichuris vulpis</i>) commonly inhabits the cecum in infected dogs; no signs are seen in light infestations; weight loss and diarrhea may become pronounced as the worm burden increases; fresh blood may accompany the feces, and anemia occasionally follows.
Cestode (tapeworm) infections	Adult cestodes such as <i>Dipylidium caninum</i> and <i>Taenia pisiformis</i> may inhabit the intestine of dogs, but rarely cause serious disease; if present, clinical signs may depend on the degree of infection, age, condition, and breed of host; clinical signs may vary from unthriftiness and malaise to colic and mild diarrhea.
Fleas	Ubiquitous blood-sucking ectoparasites, principally of dogs and cats; may cause pruritus and severe dermatological problems; act as intermediate hosts for the tapeworm <i>D. caninum</i> ; adult fleas can jump long distances and attach to potential hosts; pruritus is usually the first sign of flea infestation in dogs.
Otitis externa	Acute or chronic inflammation of the epithelium of the external auditory meatus, sometimes involving the pinna; characterized by erythema, discharge, desquamation, and varying degrees of pain and pruritis; this is the most common disease of the ear canal of dogs.

Sources: Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989; Clarence, M.F., *The Merck Veterinary Manual*, 7th edn., Merck & Co., Rahway, NJ, 1991.

SWINE (*Sus scrofa*)

Breeds

Among the strains of miniature swine available for nonclinical laboratory studies are the Göttingen minipig, the Hanford minipig, the Yucatan minipig and micropig, the Troll minipig, and the Sinclair minipig. The Göttingen minipig originated at the University of Göttingen (Germany) in the 1960s as a result of cross-breeding the Vietnamese swine, the Hormel or Minnesota miniature swine, and the German improved Landrace. Due to the small stature of the Göttingen minipig, its relatively calm demeanor, and its suitability for most testing purposes, the Göttingen minipig has rapidly become the mainstay for nonclinical testing in miniature pigs. Göttingen minipigs reach sexual maturity at a considerably earlier age than do dogs or monkeys, with boars reaching maturity at 3–4 months, and sows at 4–5 months, in comparison to dogs (7–8 months for males and ≥ 12 months for females) or

cynomolgus monkeys (3–4 years). At the age that minipigs are commonly used in toxicology studies (~3–4 months), the animals approach maturity by the end of 28-day studies, and will achieve complete maturity by the end of 13-week studies.

Transportation and Receipt

As pigs are quite sensitive animals, transportation is stressful for them, and gentle handling during transportation and at receipt is necessary. Because swine are susceptible to motion sickness, the pigs should be deprived of food for 12 h prior to transportation and during transportation unless the trip period will be many days. If the duration of transportation is long, then travel breaks should be offered to allow the animals access to water approximately every 6 h or so. Only climate-controlled vehicles should be used for transportation, especially for long trips, and where possible, the vehicle's climate should mimic the environmental conditions of the destination facility.

Within a few days of receipt, each pig should be given a thorough physical examination by a qualified veterinarian. As with the dog, this examination should include general physical appearance and behavior, assessment of heart and lung sounds, presence of external parasites, condition of gums and teeth, mobility, and presence of any abnormal secretions or signs of gastrointestinal disturbance.

Housing

At breeding facilities, swine are often housed in groups by sex. Provided sufficient space per animal is available to allow the more submissive animals to avoid the more aggressive, dominant animals, the animals should be able to live together in reasonable harmony. Stable social groups are most easily established shortly after weaning. In the laboratory facility, pigs can be housed individually or in small groups in floor pens or in cages of sufficient size. Adult boars are solitary by nature, and due to their tendency toward aggressive behavior with other males (particularly those with which the animal was not reared), strong consideration should be given to individually housing intact adult males. Boars can be group-housed if reared together or if given sufficient time to acclimate to each other under unconfined conditions in which plenty of space is available (i.e., trough space and floor space); however, the latter case is frequently impractical for most conventional laboratory facilities.

Like dogs, pigs may be housed in cages, pens, or runs, with one or more animals per primary enclosure. Primary enclosures must be well maintained with surfaces that are easily cleaned and sanitized. Floors must protect the pigs' feet and legs from injury. Best practices call for removal of excreta daily, where possible, and sanitization of pens at least weekly. Pigs should have access to clean drinking water at all times. Food is typically provided at least twice daily in most laboratory facilities, except as otherwise required to provide adequate veterinary care. Although swine can spend up to 80% of a day either lying or sleeping, during their active hours, they explore and root. If provided adequate space, swine will urinate and defecate in designated areas and maintain their specific sleeping areas in clean state. The provision of straw or other bedding greatly enhances the well-being of swine, allowing them to indulge in their normal rooting and chewing behaviors.

Minimum cage space requirements for swine are presented in Table 1.33.

Environmental Conditions

Environmental conditions recommended for swine by the *Guide* are as follows:

Room temperature: 61°F–81°F, 16°C–27°C

Relative humidity: 30%–70%

Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges stated earlier to avoid extremes and large fluctuations in these environmental variables. The *Guide* does not specify any particular lighting cycle for swine; however, a 12 h light/12 h dark cycle is

routinely used for this species. Note that the activity of laboratory swine can be more dependent on the presence of humans in the swine room than the presence or absence of light.

Physical and Physiological Parameters

Physical and physiological parameters for miniature swine are listed in Table 1.34.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Pigs (Table 1.35)

Diseases

Common diseases of pigs are briefly described in Table 1.36.

TABLE 1.33
Minimum Cage Space Requirements for Pigs^a

No. of Pigs ^a	Weight (kg)	Floor Area/Pig ft ² (m ²)
Individuals	<15	8.0 (0.72)
	≥15 to <25	12.0 (1.08)
	≥25 to <50	15.0 (1.35)
	≥50 to <100	24.0 (2.16)
2–5 in groups	<25	6.0 (0.54)
	≥25 to <50	10.0 (0.90)
	≥50 to <100	20.0 (1.80)

^a As per the National Research Council of the National Academies, 2011.

TABLE 1.34
Physical and Physiological Parameters of Miniature Swine^a

Life span	10–15 years
Adult weight	12–55 kg
Birth weight	0.4–0.7 kg
Adult food consumption	1.0–1.5 kg/day
Adult water consumption	80–120 mL/kg/day
Minimum breeding age	5–6 months
Estrus cycle (days)	14
Gestation	114 days
Weaning age	4–5 weeks
Litter size	5–8
Red cell count	5.3–9.25 × 10 ⁶ /mm ³
White cell count	4.4–26.4 × 10 ³ /mm ³
Hemoglobin	9.0–15.8 g/dL
Hematocrit	32%–61%
Platelet count	148–898 × 10 ³ /mm ³
Heart rate	83 ± 15 beats/min
Respiration rate	20 ± 9 breaths/min
Body temperature	37°C–38°C
Chromosome number	2n = 38

Source: Bollen, P.J.A. et al., *The Laboratory Swine*, CRC Press, Taylor & Francis Group, Boca Raton, FL, 2000.

^a For Gottingen and Sinclair minipigs and Yucatan micropigs.

TABLE 1.35
Identification, Bleeding, Anesthesia, and Euthanasia
Methods for Laboratory Swine

Identification methods
Cage card + plastic ear tag
Ear notch
Subcutaneously implanted transponder
Bleeding methods
Jugular, cranial caval, femoral, saphenous, and cephalic veins
Anesthesia methods
Propofol/thiopental, halothane, sevoflurane, and propofol infusion
Euthanasia methods
Barbiturates, KCl with prior general anesthesia, CO ₂ , penetrating captive bolt

TABLE 1.36
Diseases in Pigs

Disease/Health Condition	Causes
Myocarditis	Can result in increased heart rate and sudden death and may be caused by Streptococci, encephalomyocarditis virus, foot-and-mouth, vesicular disease, malignant hyperthermia, and dietary deficiencies (e.g., iron, selenium, and vitamin E).
Pericarditis	Like myocarditis, pericarditis can result in sudden death and is caused by <i>Pasteurella</i> spp., <i>Mycoplasma</i> spp., <i>Hemophilus parasuis</i> , <i>Actinobacillus pleuropneumoniae</i> , or Streptococci.
Vasculitis	Vasculitis can result in blood loss and anemia and can be caused by African swine fever, hog cholera, Erysipelas, <i>Hemophilus parasuis</i> , and <i>Actinobacillus pleuropneumoniae</i> .
Hemorrhagic conditions	Like vasculitis, hemorrhagic disorders can result in anemia and blood loss, and may be caused by a deficiency in clotting factor VIII, by African swine fever, and by platelet disorders such as thrombocytopenia.
Thrombocytopenic purpura syndrome	An immune-mediated (possibly type III hypersensitivity) thrombocytopenia (platelets <20,000/ μ L) and regenerative anemia characterized by cutaneous purpura and disseminated visceral hemorrhages.
Anemia	Under normal circumstances, anemia in swine occurs as a result of iron deficiency, parasitic infections, or gastric ulcers.
Enteritis	Enteritis results in diarrhea (ranging from watery, hemorrhagic, to mucohemorrhagic) and can be caused by various pathogens, such as coronavirus, rotavirus, <i>E. coli</i> , <i>Salmonella</i> spp., <i>Campylobacter</i> spp., and <i>Clostridium perfringens</i> .
Atrophic rhinitis	Atrophic rhinitis, characterized by nasal discharge and snout deformities, is caused by interactions between <i>Bordetella bronchiseptica</i> and toxigenic <i>Pasteurella multocida</i> .
Pneumonia	In swine, the common causes of pneumonia are <i>Mycoplasma hyopneumoniae</i> and <i>Actinobacillus pleuropneumoniae</i> .
Exudative dermatitis	Also known as "greasy pig" disease, exudative dermatitis is characterized by black greasy spots on the skin of swine and is caused by <i>Staphylococcus hyicus</i> .
Salt poisoning (Water deprivation syndrome)	Excessive intake of sodium salts or dehydration followed by rehydration creates a pathologic state of hyperosmolarity, manifested clinically by inappetence, head pressing, incoordination, blindness, circling, paddling, and/or convulsions.
Malignant hyperthermia (porcine stress syndrome)	A ryanodine receptor defect leading to unregulated release of calcium from the sarcoplasmic reticulum, excessive myofiber contraction, and increased body temperature; triggered by inhaled anesthetics or stress in pigs and humans.

Sources: Bollen, P.J.A. et al., *The Laboratory Swine*, CRC Press, Taylor & Francis Group, Boca Raton, FL, 2000; McGavin, M.D. and Zachary, J.F., *Pathologic Basis of Veterinary Disease*, Mosby, Inc., St. Louis, MO, 2007.

NONHUMAN PRIMATES

Species

Within the order Primates is the suborder Anthropoidea, which includes two nonhuman primate (NHP) infraorders, the Platyrrhine or New World monkeys and the Catarrhine or Old World monkeys. New World monkeys include the

Callitrichidae (e.g., marmosets and tamarins) and the Cebidae (e.g., howler monkeys, woolly monkeys, spider monkeys, squirrel monkeys, and owl monkeys). The most commonly used New World monkeys in laboratory facilities include the common or tufted marmoset (*Callithrix jacchus jacchus*), the cotton-top tamarin (*Sanguinus oedipus oedipus*), the moustached tamarin (*Sanguinus mystax*),

the owl monkey (*Aotus trivirgatus*), the squirrel monkey (*Saimiri* spp.), and the capuchin Monkey (*Cebus* spp.). The Old World monkeys are found in Africa and Asia. The Old World monkeys tend to be larger than their New World counterparts, and many are primarily terrestrial rather than arboreal. The Old World monkeys most commonly used in biomedical research belong to the family Cercopithecinae and include macaques, baboons, guenons, and mangabeys. Among nonhuman primates, macaques have the greatest geographic distribution. The most common macaques used in biomedical research are the cynomolgus monkey (*Macaca fascicularis*), also known as the crab-eating macaque, and the rhesus monkey (*Macaca mulatta*). Less frequently used in biomedical research are the baboons (*Papio* spp), due to the relatively large size which can only be accommodated by a select few laboratory facilities. Larger still are the chimpanzees (*Pan troglodytes*), which are actually members of the great apes, family Pongidae. In North American nonclinical testing facilities, Old World monkeys (primarily cynomolgus or rhesus monkeys) tend to be the preferred NHP models, whereas in the United Kingdom, New World monkeys are used more often than in North America.

Housing

Unlike mice and pigs, for which social groups (particularly for males) are best established during rearing, social groups for monkeys are more readily established among animals with no prior history with each other to avoid the establishment of rival factions. As with other species, the removal of dominant animals from a social group can result in fighting among lower-ranking animals in an attempt to reestablish the dominance hierarchy. Pair housing can avoid many of these complications while still providing necessary social interaction, if sufficiently compatible pairings can be identified.

New World and Old World monkeys have behavioral differences that can influence the design of the primary enclosure. Most New World species are arboreal and require enclosures that are proportionately greater in height to facilitate their natural climbing behavior as compared to more terrestrial species, such as the Old World macaques. Perches should be available for all species, and certain species, such as marmosets and owl monkeys, require nesting boxes in addition. Cages are generally made of stainless steel and generally have mesh or slatted floors to allow excreta to fall below the floor. Cages for certain Old World monkeys (e.g., macaques) often contain a false back to facilitate the restraint of animals without removing them from the cage. Certain cage types contain chute-like areas at the front of the cage that can be closed off when the monkey is in that area to allow study procedures (e.g., blood collection) to be conducted without actual animal removal.

Minimum cage space requirements for monkeys are presented in Table 1.37.

Environmental Conditions

Environmental conditions recommended for swine by the *Guide* are as follows:

Room temperature: 64°F–84°F, 18°C–29°C

Relative humidity: 30%–70%

Room air changes: 10–15/h

Environmental controls should be set toward the middle of the room temperature and relative humidity ranges stated earlier to avoid extremes and large fluctuations in these environmental variables. The *Guide* does not specify any particular lighting cycle for NHPs; however, a 12 h light/12 h dark cycle is routinely used for this species.

Physical and Physiological Parameters

Physical and physiological parameters for Old World monkeys are listed in Table 1.38 and for New World Monkeys in Table 1.39.

Identification, Bleeding, Anesthesia, and Euthanasia Methods for Monkeys (Table 1.40)

Diseases

Common diseases of nonhuman primates are briefly described in Table 1.41. As might be expected, many of these diseases are of genuine concern in humans.

TABLE 1.37
Minimum Cage Space Requirements
for Monkeys^a

Body Weight (kg)	Floor Area/ Monkey ft ² (m ²)	Cage Height in. (cm)
≤1.5	2.1 (0.20)	30 (76.2)
>1.5 and ≤3	3.0 (0.28)	30 (76.2)
>3 and ≤10	4.3 (0.4)	30 (76.2)
>10 and ≤15	6.0 (0.56)	32 (81.3)
>15 and ≤20	8.0 (0.74)	36 (91.4)
>20 and ≤25	10 (0.93)	46 (116.8)
>25 and ≤30	15 (1.40)	46 (116.8)
>30	≥25 (≥2.32)	60 (152.4)

^a As per the National Research Council of the National Academies, 2011. The *Guide* also contains housing space recommendations for other NHP species, such as chimpanzees.

TABLE 1.38
Physical and Physiological Parameters of Old World Monkeys

Parameter	<i>Macaca fascicularis</i>	<i>Macaca mulatta</i>
Adult male weight	4–8 kg	6–11 kg
Adult female weight	2–6 kg	4–9 kg
Respiratory rate	30–54 breaths/min	30–50 breaths/min
Heart rate	115–243 bpm	92–122 bpm
Rectal temperature	37°C–39°C (98.6°F–103.1°F)	37°C–39°C (98.6°F–103.1°F)
Daily food consumption	350–550 g	400–600 g
Daily water consumption	350–550 mL	400–600 mL
Urinary excretion	150–550 mL/day	Not listed
Blood volume	55–75 mL/kg	50–96 mL/kg
Red cell count	5.3–6.3 × 10 ⁶ /mL	5.1–5.6 × 10 ⁶ /mL
White cell count	6.1–12.5 × 10 ³ /mL	4.2–8.1 × 10 ³ /mL
Hemoglobin	11.0–12.4 g/dL	12.0–13.1 g/dL
Hematocrit	33%–75%	37%–40%
Platelet count	300–512 × 10 ³ /mL	260–361 × 10 ³ /mL
Chromosome number	2n = 42	2n = 42

Source: Fortman, J.D. et al., *The Laboratory Nonhuman Primate*, CRC Press, Boca Raton, FL, 2002.

TABLE 1.40
Identification, Bleeding, Anesthesia, and Euthanasia Methods for Laboratory Monkeys

Identification methods

Tattoos

Subcutaneously implanted transponder

Bleeding methods

Femoral, cephalic, saphenous, coccygeal, and jugular veins

Anesthesia methods

Ketamine, tiletamine/zolazepam, propofol, and barbiturates

Euthanasia methods

Barbiturates

Sources: AVMA, *AVMA Guidelines on Euthanasia*, AVMA, Schaumburg, IL, 2007; Fortman, J.D. et al., *The Laboratory Nonhuman Primate*, CRC Press, Boca Raton, FL, 2002.

TABLE 1.39
Physical and Physiological Parameters of New World Monkeys

Parameter	<i>Saimiri sciureus</i>	<i>Callithrix jacchus</i>
Adult male weight	0.9–1.1 kg	0.3–0.5 kg
Adult female weight	0.7 kg	0.3–0.4 kg
Respiratory rate	55–58 breaths/min	Not listed
Heart rate	215–263 bpm	194–242 bpm
Rectal temperature	39°C–40°C (101.7°F–103.6°F)	37°C–39°C (98.2°F–101.5°F)
Daily food consumption	45–60 g	20 g plus fruit
Red cell count	6.3–7.1 × 10 ⁶ /mL	4.6–6.6 × 10 ⁶ /mL
White cell count	6.0–9.1 × 10 ³ /mL	4.9–11.3 × 10 ³ /mL
Hemoglobin	12.2–13.6 g/dL	12.6–19.6 g/dL
Hematocrit	39%–44%	32%–54%
Platelet count	Not listed	180–382 × 10 ³ /mL
Chromosome number	2n = 44	2n = 46

Source: Fortman, J.D. et al., *The Laboratory Nonhuman Primate*, CRC Press, Boca Raton, FL, 2002.

TABLE 1.41
Diseases in Nonhuman Primates (NHPs)

Disease	Causes
Measles	Caused by a human <i>Morbillivirus</i> ; the means of infection is through exposure to aerosols from affected monkeys or humans and can be best prevented via immunization. In most Old World monkeys, infections are generally asymptomatic. In contrast, infections in certain New World species (e.g., marmosets and owl monkeys) can result in fatal gastroenterocolitis.
Herpes	<i>Herpes tamarinus</i> and <i>Herpes simplex</i> can be of concern in New World species. The most sensitive species include tamarins, marmosets, and owl monkeys, whereas squirrel, capuchin, and spider monkeys serve as reservoir hosts for <i>H. tamarinus</i> , while humans serve as reservoir hosts for <i>H. simplex</i> . In susceptible species, infection can be fatal.
B virus	B virus (Cercopithecine herpesvirus 1) is an alphaherpesvirus that is endemic in Asian macaques. Most macaques are carriers of B virus without clinical signs of disease. Zoonotic infection of humans with B virus can cause fatal encephalomyelitis or severe neurologic impairment.
Tuberculosis	Caused by <i>Mycobacterium tuberculosis</i> or <i>Mycobacterium bovis</i> ; tuberculosis can be an even greater problem in monkey populations than in human populations, with Old World monkeys having an apparently greater susceptibility. Prevention is best accomplished through the acquisition of animals from vendors that employ careful colony health management and by tuberculin skin testing of both animals and humans that come into contact with the animals.
Salmonellosis	Caused by <i>Salmonella enteritidis</i> and <i>Salmonella typhimurium</i> . May occur by exposure to feed contaminated by rodent feces or via exposure to human carriers. Signs are similar to shigellosis albeit milder, and include diarrhea generally without mucus or blood. Can be fatal in severe cases.
Pneumonia	In NHPs, the common causes of pneumonia are <i>Streptococcus pneumoniae</i> , <i>Klebsiella pneumoniae</i> , and <i>Bordetella bronchiseptica</i> . The clinical signs resemble those of human disease (cough and dyspnea), although <i>Streptococcus pneumoniae</i> can also cause meningitis. The diseases can be successfully treated with penicillins and cephalosporins.
Shigellosis	The most common member of <i>Shigella</i> spp. encountered in NHPs is <i>Shigella flexneri</i> . Clinical signs can include diarrhea, dehydration, fatigue, anorexia, and weight loss, with indications of abdominal pain. Supportive care is warranted where necessary, and the disease can be treated with enrofloxacin.
Campylobacteriosis	The most common species that cause Campylobacteriosis in NHPs are <i>Campylobacter jejuni</i> and <i>Campylobacter coli</i> . Common clinical signs include watery diarrhea, dehydration, and weight loss. The disease has been successfully managed using erythromycin.
Monkeypox	Monkeypox is caused by <i>Orthopoxvirus</i> and affects Old World and New World primates. In NHPs, monkeypox presents as a rash that can last for 4–6 weeks. Fatalities are rare, but can occur in infant monkeys. Humans can be infected by direct contact with a monkey's blood or bodily fluids or via an animal bite.
Pulmonary Acariasis	Caused by parasitism of the respiratory tract tissue by <i>Pneumonyssus simicola</i> , primarily in Old World monkeys. Infections are generally asymptomatic, but may result in coughing or sneezing. Can be managed with s.c. ivermectin.
Balantidiasis	Caused by the ciliated protozoan <i>Balantidium coli</i> , found in Old World monkeys, and generally transmitted via the fecal–oral route. Balantidiasis infections may be subclinical but can also result in diarrhea and ulcerative colitis.

Sources: Fortman, J.D. et al., *The Laboratory Nonhuman Primate*, CRC Press, Boca Raton, FL, 2002; Cho, C.T. and Wenner, H.A., *Bacteriol. Rev.*, 37, 1, 1973; Huff, J.L. and Barry, P.A., *Emerg. Infect. Dis.*, 9(2), 246, 2003; Takasaka, M. et al., *Jpn. J. Med. Sci. Biol.*, 41, 1–13, 1988; Nakauchi, K., *J. Vet. Med. Sci.*, 61(1), 63, 1999.

REFERENCES

- Code of Federal Regulations (CFR), Title 9; Subchapter A, Animal Welfare, Office of the Federal Register, Washington, DC, 1985.
- National Research Council of the National Academies, *Guide for the Care and Use of Laboratory Animals*, 8th edn., The National Academies Press, Washington, DC, 2011.
- Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, *Recognition and Alleviation of Pain and Distress in Laboratory Animals*, National Academy Press, Washington, DC, 1992, pp. 3–7.
- Russell, W.M.S. and Burch, R.L., *The Principles of Humane Experimental Techniques*, Methuen & Co., London, U.K., 1959.
- Evans, I.E. and Maltby, C.J., *Technical Laboratory Animal Management*, MTM Associates, Manassas, VA, 1989.
- AVMA, *AVMA Guidelines on Euthanasia*, AVMA, Schaumburg, IL, 2007.
- Williams, C.S.F., *Practical Guide to Laboratory Animals*, C.V. Mosby, St. Louis, MO, 1976.
- LAMA Lines*, Newsletter of the Laboratory Animal Management Association, 4, September/October, 1988.
- Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, *Infectious Diseases of Mice and Rats*, National Academy Press, Washington, DC, 1991.
- Clarence, M.F., *The Merck Veterinary Manual*, 7th edn., Merck & Co., Rahway, NJ, 1991.
- Benenson, A.S., Ed., *Control of Communicable Diseases Manual*, 16th edn., American Public Health Association, Washington, DC, 1995.
- Terril, L.A. and Clemons, D.J., *The Laboratory Guinea Pig*, CRC Press, Boca Raton, FL, 1998.

13. Field, G. and Jackson, T.A., *The Laboratory Canine*, CRC Press, Taylor & Francis Group, Boca Raton, FL, 2007.
14. Bollen, P.J.A., Hansen, A.K., and Rasmussen, H.J., *The Laboratory Swine*, CRC Press, Taylor & Francis Group, Boca Raton, FL, 2000.
15. Fortman, J.D., Hewett, T.A., and Bennett, B.T., *The Laboratory Nonhuman Primate*, CRC Press, Boca Raton, FL, 2002.
16. Robinson, S., Chapman, K., Hudson, S., Sparrow, S., Spencer-Briggs, D., Danks, A., Hill, R., Everett, D., Mulier, B., Old, S., and Bruce, C., *Guidance on Dose Level Selection for Regulatory General Toxicology Studies for Pharmaceuticals*, Laboratory Animal Science Association and National Centre for the Replacement, Refinement, and Reduction of Animals in Research, December, 2009.
17. McGavin, M.D. and Zachary, J.F., *Pathologic Basis of Veterinary Disease*, Mosby, Inc., St. Louis, MO, 2007.
18. Cho, C.T. and Wenner, H.A., Monkeypox virus. *Bacteriol. Rev.* 37: 1–18, 1973.
19. Huff, J.L. and Barry, P.A., B-virus (Cercopithecine herpesvirus 1) infection in humans and macaques: Potential for zoonotic disease. *Emerg. Infect. Dis.* 9(2): 246–250, 2003.
20. Takasaka, M., Kohno, A., Sakakibara, I., Narita, H., and Honjo, S., An outbreak of salmonellosis in newly imported cynomolgus monkeys. *Jpn. J. Med. Sci. Biol.* 41: 1–13, 1988.
21. Nakauchi, K., The prevalence of *Balantidium coli* infection in fifty-six mammalian species. *J. Vet. Med. Sci.* 61(1): 63–65, 1999.
22. Borchard, R.E. et al., *Drug Dosage in Laboratory Animals. A Handbook*, 3rd edn., CRC Press, Boca Raton, FL, 1992.

APPENDIX: ADDITIONAL RELATED INFORMATION (TABLES 1.A.1 THROUGH 1.A.4)

TABLE 1.A.1

Guiding Principles in Use of Animals in Toxicology

1. The use, care, and transportation of animals for training and for toxicological research and testing for the purpose of protecting human and animal health and the environment must comply with all applicable animal welfare laws.
2. When scientifically appropriate, alternative procedures that reduce the number of animals used, refine the use of whole animals, or replace whole animals (e.g., *in vitro* models, invertebrate organisms) should be considered.
3. For research requiring the use of animals, the species should be carefully selected and the number of animals kept to the minimum required to achieve scientifically valid results.
4. All reasonable steps should be taken to avoid or minimize discomfort, distress, or pain of animals.
5. Appropriate aseptic technique, anesthesia, and postoperative analgesia should be provided if a surgical procedure is required. Muscle relaxants or paralytics are not to be used in place of anesthetics.
6. Care and handling of all animals used for research purposes must be directed by veterinarians or other individuals trained and experienced in the proper care, handling, and use of the species being maintained or studied. Veterinary care is to be provided in a timely manner when needed.
7. Investigators and other personnel shall be qualified and trained appropriately for conducting procedures on living animals, including training in the proper and humane care and use of laboratory animals.

TABLE 1.A.1 (continued)

Guiding Principles in Use of Animals in Toxicology

8. Protocols involving the use of animals are to be reviewed and approved by an IACUC before being initiated. The composition and function of the committee shall be in compliance with applicable animal welfare laws, regulations, guidelines, and policies.
9. Euthanasia shall be conducted according to the most current guidelines of the American Veterinary Medical Association (AVMA) Panel on Euthanasia or similar bodies in different countries.

Source: Society of Toxicology, 1999. With permission.

TABLE 1.A.2

General Information Sources for Care and Use of Research Animals

1. *Public Health Service Policy on Humane Care and Use of Laboratory Animals*. PHS (Public Health Service), 1996, U.S. Department of Health and Human Services, Washington, DC 22 pp. [PL 99-158. Health Research Extension Act, 1985].
2. The Animal Welfare Act of 1966 (P.L. 89-544) as amended by the Animal Welfare Act of 1970 (P.L. 91-579); 1976 Amendments to the Animal Welfare Act (P.L. 94-279); the Food Security Act of 1985 (P.L. 99-198), Subtitle F (Animal Welfare File Name: PL99198); and the Food and Agriculture Conservation and Trade Act of 1990 (P.L. 101-624), Section 2503, Protection of Pets (File Name: PL 101624). Rules and regulations pertaining to implementation are published in the Code of Federal Regulations, Title 9 (Animals and Animal Products), Chapter 1, Subchapter A (Animal Welfare). Available from Regulatory Enforcement and Animal Care, APHIS, USDA, Unit 85, 4700 River Road, Riverdale, MD 20737-1234, File Name 9CFR93. www.nal.usda.gov/awic/legislat/awicregs.html
3. *Guide for the Care and Use of Laboratory Animals*. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, DC, 1996 or succeeding revised editions. www.nap.edu/readingroom/books/labrats
4. *International Guiding Principles for Biomedical Research Involving Animals*. Council for International Organizations of Medical Sciences (CIOMS), Geneva, 1985.
5. *Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education*. Ad Hoc Animal Research Committee, New York Academy of Sciences, 1988.
6. *Recognition and Alleviation of Pain and Distress in Laboratory Animals. A report of the Institute of Laboratory Animal Resources Committee on Pain and Distress in Laboratory Animals*. NCR (National Research Council). Washington, DC: National Academy Press, 1992.
7. *Education and Training in the Care and Use of Laboratory Animals: A Guide for Developing Institutional Programs*. AVMA (American Veterinary Medical Association). Report of the AVMA panel on euthanasia. *J. Am. Vet. Med. Assoc.* 218(5), 669–696, 2001.
8. *Guide to the Care and Use of Experimental Animals*. CCAC (Canadian Council on Animal Care), Vol. 1, 2nd ed., Edited by E. D. Olfert, B. M. Cross, and A. A. McWilliam. Ontario, Canada: Canadian Council on Animal Care, 1993. 211 pp.

Source: Compiled by the Society of Toxicology.

TABLE 1.A.3
Commonly Used Anesthetics

This table lists injectable anesthetics and preanesthetics used in various animal species. The medications are sometimes used in combination to produce anesthesia. These combinations are indicated by brackets where applicable. For example, atropine, morphine, and pentobarbital can be used in combination in dogs. Atropine and morphine are administered 30 min before pentobarbital to reduce parasympathetic secretions and to provide analgesia. The routes of exposure listed are those generally used. Other routes of administration may be used for some of the medications. The reader should consult the reference for information on alternate routes and dosage information.

Species	Anesthetic	Time ^a (min)	Route ^b	Dose (mg/kg)
Mouse	Amobarbital		IV	54
	Barbital		IV	234
	Chloral hydrate		IP	400
	α-Chloralose		IP	114
	Chlorobutanol (in 50% alcohol)		IP	175
	Droperidol (2%)-Fentanyl (0.04%)		IP	0.02–0.05 ^c
	Etomidate		IP	22–25
	Hexobarbital		IV	47
	Ketamine		IV	50
	Ketamine		IP	100–200
	Ketamine		IM	400
	Pentobarbital		IV	35
	Phenobarbital		IV	134
	Probarbital		IP	75
	Secobarbital		IV	30
	Thiamylal		IV	25–50
	Thiopental		IV	25
	Tribromoethanol		IV	120
	Urethane		IP	1500
Rat	[Acepromazine		IP	12]
	[Ketamine		IP	120]
	Amobarbital		IV	55
	Barbital		IP	190
	Chloral hydrate		IP	300
	α-Chloralose		IP	55
	α-Chloralose		IV	100
	Diallylbarbituric acid		SC	60
	Droperidol (2%)-Fentanyl (0.04%)		IM	0.3 ^c
	Droperidol (2%)-Fentanyl (0.04%)		IP	0.13 ^c
	Hexobarbital		IP	75
	Inactin		IP	100
	Ketamine		IP	100
	Ketamine		IM	100
	Ketamine		IP	40–160
	Methohexital		IP	37.5
	Pentobarbital		IV	25
	[Pentobarbital		IP	35]
	[Chloral hydrate		IP	160]

TABLE 1.A.3 (continued)
Commonly Used Anesthetics

Species	Anesthetic	Time ^a (min)	Route ^b	Dose (mg/kg)
Guinea pig	[Pentobarbital		IP	10]
	[Ketamine		IP	75]
	Phenobarbital		IP	40
	Phenobarbital		IV	100
	Probarbital		SC	225
	Secobarbital		IV	17.5
	Thiopental		IV	25
	Tiletamine-Zolazepam (1:1)		IM	20–30
	Tribromoethanol		IP	550
	Urethane		IP	780
	Urethane		SC	1200
	[Xylazine		IM	6]
	[Ketamine		IM	80]
	Amobarbital		IV	50
	Chloral hydrate		IP	400
Rabbit	Chlorobutanol (in 50% alcohol)		IP	175
	Droperidol (2%)-Fentanyl (0.04%)		IM	0.66–0.88 ^c
	Droperidol (2%)-Fentanyl (0.04%)		IP	0.8
	Ketamine		IM	44–256
	Pentobarbital		IP	30
	Pentobarbital		IM	15–30
	Pentobarbital		IV	30
	[Pentobarbital		IP	35]
	[Chloral hydrate		IP	160]
	Phenobarbital		IP	100
	Secobarbital		IV	20
	Thiopental		IV	20
	Tribromoethanol		IV	100
	Urethane		IP	1500
	Amobarbital		IV	40
	Barbital		IV	175
	α-Chloralose		IV	120
	Chloral hydrate		IV	200
	Diallylbarbituric acid		IV	50
	Droperidol (2%)-Fentanyl (0.04%)		IM	0.22 ^c
	Hexobarbital		IV	25
	Ketamine		IV	15–20
	Ketamine		IM	44
	[Morphine	30	SC	10]
	[Chlorobutanol (in 50% alcohol)		PO	175]
	Paraldehyde		IV	300
	Pentobarbital		IV	30
	Pentobarbital		IP	40
	Pentobarbital		IV	25–40
	Phenobarbital		IV	200

(continued)

TABLE 1.A.3 (continued)
Commonly Used Anesthetics

Species	Anesthetic	Time ^a (min)	Route ^b	Dose (mg/kg)
Cat	Probarbital		IP	66
	Secobarbital		IV	22.5
	Thiamylal		IV,IP	45–50
	Thiopental		IV	20
	Tribromoethanol		IV	80
	Urethane		IV	1000
	[Urethane		IP	700]
	[Pentobarbital		IP	40]
	Xylazine		IM	5
	[Xylazine		IM	5]
	[Ketamine		IM	50]
	Amobarbital		IV	11
	Barbital		IV	200
	Chloral Hydrate		PO	250
	α -Chloralose		IV	75
	[α -Chloralose		IV	50]
	[Urethane		IV	50]
	[α -Chloralose		IV	80]
	[Pentobarbital		IV	12]
	[α -Chloralose		IV	80]
	[Pentobarbital		IV	6]
	Diallylbarbituric acid		IV	36
	Hexobarbital		IV	25
	Ketamine		IM	11–33
	Ketamine		IV	11–22
	[Ketamine		IM	6.6–22]
	[Acepromazine		IV,IM	0.22–0.55]
	[Ketamine		IM	6.6–22]
	[Diazepam		IV,IM	0.33–1.1]
	[Ketamine	10–15	IM	10–15]
	[Pentobarbital		IV	30]
	[Ketamine		IM	6.6–22]
	[Xylazine		IM	0.44]
	[Pentobarbital		IP	20]
	[Barbital		IP	200]
	Methohexital		IV	11
	Paraldehyde		IV	300
	Pentobarbital		IV	25
	Phenobarbital		IP	180
	Secobarbital		IV	25
	Thiamylal		IV	17.6
	Thiamylal		i.thoracic	25
	Thiopental		IV	28
	Tiletamine-Zolazepam (1:1)		IM	6–13
	Tribromoethanol		IV	100
	Urethane		IV	1250
	[Urethane		IP	400]
	[α -Chloralose		IP	50]
	[Urethane		IP	280]
	[Diallylbarbituric acid		IP	70]

TABLE 1.A.3 (continued)
Commonly Used Anesthetics

Species	Anesthetic	Time ^a (min)	Route ^b	Dose (mg/kg)
Dog	[Urethane		IP	360]
	[Diallylbarbituric acid		IP	90]
	[Urethane		IP	250]
	[Pentobarbital		IP	30]
	[Acepromazine	10–15	IM	0.55]
	[Ketamine	5	IM	11–22]
	[Thiamylal		IV	To effect]
	Amobarbital		IV	50
	Barbital		IV	220
	[Barbital		IV	250]
	[Thiopental		IV	15]
	[Barbital		IV	220]
	[Pentobarbital		IV	15]
	Chloral hydrate		IV	125
	α -Chloralose		IV	100
	Dial-urethane		IV	10 ^c
	Droperidol (2%)-Fentanyl (0.04%)		IM	0.05–0.15 ^c
	Droperidol (2%)-Fentanyl (0.04%)		IV	0.03–0.09 ^c
	Etomidate		IV	1.5–3
	[α -Chloralose		IV	100]
	[α -Chloralose		IV	0.17 ^d]
	[Morphine		IM	2]
	[α -Chloralose		IV	100]
	[Morphine	30	SC	1]
	[α -Chloralose		IV	100]
	[Morphine	60	SC	1]
	[α -Chloralose		IV	80]
	[α -Chloralose		IV	50]
	[Thiopental		IV	15]
	[Morphine	30	SC	10]
	[Chlorobutonal (in 50% alcohol)		PO	225]
	Morphine		SC	1
	Thiopental		IV	20
	Hexobarbital		IV	30
	Methohexital		IV	11
	Paraldehyde		IV	300
	[Pentobarbital		IP	30]
	[Pentobarbital		IV	6 ^d]
	[Pentobarbital		IV	10]
	[α -Chloralose		IV	80]
	[Morphine	30	SC	10]
	[Pentobarbital		IV	20]
	[Morphine	30	IM	2]
	[Pentobarbital		IV	15]
	[Morphine	60	IM	3]
	[Pentobarbital		IV	12]

TABLE 1.A.3 (continued)
Commonly Used Anesthetics

Species	Anesthetic	Time ^a (min)	Route ^b	Dose (mg/kg)
Monkey	Atropine	30	SC	1
	Morphine	30	SC	10
	Pentobarbital		IV	30
	Phenobarbital		IV	80
	Phenobarbital		IV	200
	Thiopental		IV	15
	Promazine	5	IV,IM	4.4
	Ketamine		IM	17.6
	Promazine	5	IV,IM	4.4
	Ketamine		IV	To effect
	Secobarbital		PO	40
	Thiamylal		IV	17.6
	Tiletamine-Zolazepam (1:1)		IM	6–13
	Thiopental		IV	25
	Tribromoethanol		IV	125
	Urethane		IV	1000
	Urethane		IP	500
	α -Chloralose		IP	50
	Urethane		IV	480
	α -Chloralose		IV	48
	Morphine		IV	2
	Morphine	60	SC	2
	Urethane		IV	250
	α -Chloralose		IV	60
	Morphine	60	SC	3
	Urethane		IV	50
	α -Chloralose		IV	13
	Diallylbarbituric acid		IV	8
	Morphine	30	SC	5
	Urethane		PO	1500
	Xylazine	5	IV	1
	Ketamine		IV	10
	Amobarbital		IV	40
	Dial-Urethane		IV	0.7 ^c
	Droperidol (2%)-Fentanyl (0.04%)		IM	0.11 ^c
	Ketamine		IV	28–45
	Ketamine		IM	7–40

TABLE 1.A.3 (continued)
Commonly Used Anesthetics

Species	Anesthetic	Time ^a (min)	Route ^b	Dose (mg/kg)
	Ketamine	10–15	IM	18
	Thiamylal		IV	15
	Pentobarbital		IV	20–33
	Pentobarbital		IP	30
	Pentobarbital		IV	25
	Phenobarbital		IP	100
	Secobarbital		IV	17.5
	Tiletamine-Zolazepam (1:1)		IM	3
	Thiamylal		IV	25
	Xylazine	10–15	IM	6
	Ketamine		IV	To effect
	Xylazine		IM	6
	Ketamine		IM	7–40

Source: Borchard, R.E. et al., *Drug Dosage in Laboratory Animals. A Handbook*, 3rd edn., CRC Press, Boca Raton, FL, 1992. With permission.

^a The numbers refer to time elapsed (in min) before the injection of the following drug.

^b Most common route of administration. IM, intramuscular; IP, intraperitoneal; IV, intravenous; PO, per os (orally).

^c Dose is given as mL/kg.

^d mg/kg/min infusion.

TABLE 1.A.4
Advantages and Disadvantages of Anesthetic Agents and Adjuncts

Agent	Advantages	Disadvantages
1. Injection anesthetics		
α-Chloralose	Inexpensive, easy to use, rapid onset. Less reflex depression than barbiturates. Catecholamine release may support circulation.	Constantly changing anesthetic state. Poor reproducibility. Low water solubility. Inject warm or in a 10% solution with propylene glycol 200.
Dial (Ciba) ^a	More rapid onset and less toxic than urethane alone. Less reflex depression than with barbiturate alone.	Urethane toxicity limits use to acute experiment.
Droperidol-Fentanyl	IV use not generally required. Analgesic; can antagonize opioid component (fentanyl).	Cardiac and respiratory depression. Transient behavioral changes. Thermoregulatory upset; vomiting, defecation.
Etomidate	Potent hypnotic, wide margin of safety, rapid induction.	Not analgesic in subanesthetic doses.
Ketamine	IV use not generally required. Large margin of safety; no cardiovascular nor respiratory depression. Analgesic.	Poor relaxation and poor recovery; convulsive, hallucinogenic. Retention of reflexes. Used in combination with phenothiazines, benzodiazepines, and xylazine to overcome disadvantages.
Methohexital	Very short duration (<15 min, may be a disadvantage). Recovery from metabolism (use in low-fat animal).	IV use required; short duration; violent recovery possible. Metabolism required; low margin of safety.
Pentobarbital	Rapid onset. High water solubility.	IV use required generally. Marked cardiovascular and reflex depression. Extravascular → severe inflammation, sloughing.
Thiamylal	Short duration (15–30 min). Recovery from redistribution (metabolism later). Good anesthesia and relaxation.	Longer duration in animals with low body fat. IV use generally required. Cardiovascular and respiratory depression; low margin of safety.
Thiopental	Short duration (15–30 min). Useful to include anesthesia prior to inhalation agents.	Solution rapidly decomposes. Fat solubility → remains in the body for a long time.
Tiletamine–Zolazepam	Similar to ketamine–diazepam combinations.	
Urethane	Little reflex depression. High water solubility. Long duration.	Liver and bone marrow toxicity. Used only in acute experiments.
2. Inhalation anesthetics	Constant and reproducible anesthesia; therefore, less data variability.	More skill required. Some need expensive equipment. Alveolar tension difficult to monitor in animals smaller than rat.
Cyclopropane	Rapid induction. Easy to measure—as difference from oxygen.	Explosive, circulatory adaptation. Tendency to laryngospasm.
Diethyl ether	Respiratory stimulation. Good muscle relaxation.	Slow induction and slow recovery. Explosive and stimulates secretions. Long duration in body fat. Circulatory adaptation occurs.
Floroxene	Cardiovascular stimulation. Little respiratory depression.	Irritating and explosive over 4%. Circulatory adaptation. Metabolized in the body.
Halothane	Rapid induction, rapid recovery. Nonexplosive and potent.	Expensive. Cardiovascular depression and adaptation. Sensitization of myocardium to catecholamines. Poor analgesia; metabolized. Need precision vaporizers. Malignant hyperthermia implications.
Isoflurane	Low metabolism. Nonexplosive. No circulatory adaptation → “constant” anesthetic state. Rapid induction, rapid recovery.	Expensive. Cardiorespiratory depression. Need precision vaporizers.
Methoxyflurane	Potent and nonexplosive. Nonirritating to respiratory tract. Good analgesia (postanesthetic) and muscle relaxation. Precision vaporizers not needed.	Slow induction and slow recovery. Alveolar–arterial gradient. High fat solubility → long duration in body. High oxygen flows to vaporize. Highly metabolized. Ages rubber equipment.
Nitrous oxide	Not metabolized. Easily measured and used. Analgesic, additive to other anesthetics.	Only analgesia in safe concentrations. Need muscle relaxants to prevent movement. Hypoxia and diffusion hypoxia.
3. Adjuncts to anesthesia	Facilitate induction and maintenance of anesthesia; improve safety.	Injectable drugs, and not advised for acute experiments.
Atropine	Decreases respiratory secretion and vagal bradycardia.	Ganglionic blockade and CNS effects.
Benzodiazepine tranquilizers (Diazepam)	Used with several anesthetics, especially dissociative types (e.g., ketamine). Improves induction and recovery; better relaxation (allows intubation).	

TABLE 1.A.4 (continued)
Advantages and Disadvantages of Anesthetic Agents and Adjuncts

Agent	Advantages	Disadvantages
Phenothazine tranquilizers (Chlorpromazine)	Preanesthetic sedation. Reduces dose of anesthetic required.	Many pharmacological actions, especially cardiovascular depression. Prolonged recovery.
Curare	Immobility with minimal anesthesia or with nitrous oxide. "Ordinary doses" do not enter brain.	Release histamine. Need artificial ventilation. Must use analgesics.
Succinylcholine	Rapid onset and short duration. Can titrate IV. Immobility with minimal anesthesia.	Increased Serum K ⁺ . Implicated in malignant hyperthermia. Other cholinergic actions. Must use analgesics.
Narcotics (morphine)	Analgesia and sedation facilitate anesthesia. Use as only anesthetic if support ventilation.	Release histamine. Respiratory depression causing delay in induction of inhalation anesthesia.
Xylazine	Preanesthetic sedation; reduces dose of anesthetic; analgesic. Emetic action (especially in cats; may be a disadvantage). Antagonized by α_2 blockers (e.g., yohimbine)	Cardiorespiratory depression; emesis; severe CNS depression.

Source: Borchard, R.E. et al., *Drug Dosage in Laboratory Animals. A Handbook*, 3rd edn., CRC Press, Boca Raton, FL, 1992. With permission.

^a Contains urethane, 400 mg/mL; diallyl barbituric acid, 100 mg/mL.

2 General Toxicology

Carol S. Auletta, DABT, RAC, MBA

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INTRODUCTION

This chapter presents information on designing and implementing preliminary and repeated-dose (subchronic and chronic) toxicity studies and on interpreting results of these studies. Suggested study designs are provided to present an overview of typical studies of various duration in rodent and nonrodent species. A variety of government guidelines and regulations for study design have been published; several of these are summarized in the tables presented in this chapter. Samples of much of the documentation necessary to implement a study in our laboratory, as well as checklists to confirm that all appropriate prestudy tasks have been performed, are presented for guidance in the type of information required to initiate a toxicity study. Tables presenting guidelines for many of the logistical and scientific decisions which must be made to select appropriate doses, dose volumes, dosing apparatus, and dose administration procedures summarize several formal and informal “rules” used in our and other laboratories. Formulas and sample calculations are provided for calculating doses, dietary concentrations, and test material requirements. Guidelines for types of clinical signs and their experimental significance, as well as historical control values for rodent body weight, and survival in our laboratory, are provided for use in evaluating experimental results and interpreting the data. One particularly useful table summarizes the types of organ weight changes which would be expected as a result of food deprivation and resultant decreases in body weight gain; this is helpful in differentiating truly toxic effects from those which are secondary to decreases in weight gain associated with palatability problems or other causes.

It is important to remember that the information and guidelines provided are basic, general suggestions which will apply in many situations but which must be reviewed carefully to assure that they address specific needs. The toxicologist designing and interpreting a study will have information on scientific and regulatory concerns which may require modification of “standard” designs and procedures. It should also be noted that some of the information

is specific to our laboratory and, although it should provide useful guidance, it may not be completely transferable to other situations.

SAMPLE STUDY DESIGNS

Table 2.1 presents a summary of study design components for reference. Tables 2.2 and 2.3 provide specifics of suggested designs for a combination of MTD (maximum tolerated dose) and range-finding studies designed to obtain a maximum amount of information with the use of a minimal number of animals. The MTD/dose range-finding study in nonrodents is a design proposed by a working group of 12 pharmaceutical companies in Europe and is considered to be an optimized design that reflects current best practices and reduces the number of animals used without compromising the science and usefulness of this study in selecting doses for the subsequent study.¹

Acute toxicity studies are no longer recommended for development of pharmaceuticals. ICH Guidance for Industry M3(R2), Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (2010) states: “Historically, acute toxicity information has been obtained from a single-dose toxicity study in two mammalian species using both the clinical and a parenteral route of administration. However, such information can be obtained from appropriately conducted dose-escalation studies or short duration range-finding studies that define an MTD in the general test species. When this acute toxicity information is available from any study, separate single-dose studies are not recommended.” In some cases, regulatory agencies may still require acute studies, so it is prudent to confirm this. Acute toxicity studies are generally still mandated for testing of chemicals. A variety of options using fewer animals have been established. LD₅₀ studies are no longer performed.

Study designs for combination of MTD and range-finding studies performed routinely in our laboratory are presented in Tables 2.2 and 2.3. These are usually adequate to obtain acute toxicity information.

TABLE 2.1
Standard Toxicity Study Designs

Species	Duration	No/Sex	Age/Weight at Dosing	Recovery	No/Sex	TK (Control—2 Times)	Clin Path	Ophth	ECG	Organ wts	Histo
Rat	MTD/7 days	2/5	≥6 weeks	No	—	Option	Term – 7 days	—	—	—	None
	7-day mg fnd	5	10 weeks	No	—	Days 1 and 7	Term	—	—	—	Limited
	14 or 28 days	10	8–10 weeks	Option—1–2 weeks	5	Days 1 and Term	Term	Pre/Term	—	X	C & H
	3 months	10	~6 weeks	Option—1 month	5	Day 1, 3 months	Term	Pre/Term	—	X	C & H
	6 months	15	~6 weeks	Option	5	Day 1 and 6 months	Term	Pre/Term	—	X	C & H
Mouse	MTD/7 day	2/5	≥6 weeks	No	—	Option	Term – 7 days	—	—	—	None
	7 day mg fnd	6	6–8 weeks	No	—	Day 1 and 7	Term 3/3	—	—	—	Limited
	14 or 28 days	10	6–8 weeks	Option—1–2 weeks	5	Day 1 and Term	Term 5/5	Pre/Term	—	X	C & H
	3 months	10	6–8 weeks	Option—1 month	5	Day 1, 3 months	Term 5/5	Pre/Term	—	X	C & H
Dog	MTD/7 day	1/2	Any	No	—	Option	Pre/Term 7 days	—	—	—	Optional
	7 day mg fnd	1	Any	No	—	Days 1 and 7	Pre and term	—	—	No	Limited
	3 months	4	7–8 months	Option—1 month	2	Day 1, 3 months	Pre and term	Pre and term	Pre and term	X	All
	9 months	4	5–6 months	No	2	Day 1, 6 and 9 months	Pre, 6 and 9 months	Pre and term	Pre and term	X	All
Monkey	12 months	4	5–6 months	No	2	Day 1, 6 and 12 months	Pre, 6 and 12 months	Pre and term	Pre and term	X	All
	MTD/7 day	1/2	≥2 kg	No	—	Option	Pre/Term – 7 days	—	—	—	Optional
	7 day mg fnd	1	≥2 kg	No	—	Days 1 and 7	Pre and term	—	—	No	Limited
	3 months	4	≥2 kg	Option—1 months	2	Day 1, 3 months	Pre and term	Pre and term	Pre and term	X	All
	9 months	4	≥2 kg	No	2	Day 1, 6 and 9 months	Pre, 6 and 9 months	Pre and term	Pre and term	X	All
Minipig	12 months	4	≥2 kg	No	2	Day 1, 6 and 12 months	Pre, 6 and 12 months	Pre and term	Pre and term	X	All
	MTD/7 days	1/2	Any	No	—	Option	Pre/Term – 7 days	—	—	—	Optional
	14 or 28 days	3	3–4 months	Option—1–2 weeks	2	Day 1 and term	Pre and term	Pre and term	Pre and term	X	All
	3 months	4	3–4 months	Option—1 month	2	Day 1 and Term	Pre and term	Pre and term	Pre and term	X	All
	9 months	4	3–4 months	No	2	Day 1, 6, and 9 months	Pre, 6 and 9 months	Pre and term	Pre and term	X	All

Notes: Numbers do not include TK Satellite groups. For mice Clin Path- 3/3 or 5/5 - 3 or 5/sex Hem, 3 or 5/sex-clin chem; Rodent MTD four groups of 2/2; 7-day-3 groups of 5/5 (control and two treated groups). Standard no. of groups—four (control and three treated groups) unless noted. Recovery—control (C) and high-dose (H) groups. Dermal studies often require two controls (untreated and vehicle-treated). ECG's pretest and ~ t max during study.

TABLE 2.2**MTD/Repeat-Dose Range-Finding Toxicity Study in Rodents (Non-GLP)**

Duration	<ul style="list-style-type: none"> Up to 3 weeks
Species /age at treatment initiation	<ul style="list-style-type: none"> Albino rats or mice, 6 weeks or older
No. of animals	<ul style="list-style-type: none"> Phase 1–16 (four groups of two males and two females) Phase 2–30 (three groups of five males and five females) Toxicokinetics—Phase 2: 24 (6/sex/treatment group)
Dose procedure	<ul style="list-style-type: none"> MTD: The test article will be administered to two males and two females per dose in up to four single doses, each followed by a 3- or 4-day post-dose observation period. The dose will be increased or decreased for each subsequent group, based on responses to preceding doses, until dose-limiting toxicity is observed (MTD), after which no additional doses will be administered. If no MTD is established after four dose cycles, the Sponsor will be consulted for a decision on whether additional doses should be administered. Repeat dose: After the MTD has been established, the MTD and a lower dose, or other doses selected in consultation with the Sponsor, will be administered to 5 rats/sex/dose for 7 consecutive days. A control group of 5/sex will receive vehicle
Observations	<ul style="list-style-type: none"> Clinical signs: daily Physical examinations: MTD: prior to each dose, 3–4 days following the final dose. Repeat dose: pretest and termination. Body weights: MTD: prior to each dose, 3–4 days following final dose Repeat dose: pretest, Day 3, and termination. Food consumption: Repeat dose: pretest, Day 3, and termination. Hematology, clinical chemistry: termination—all animals in the repeat-dose phase
Toxicokinetics (optional)	<ul style="list-style-type: none"> Sampling: Phase 2: Following the first and last doses (Days 1 and 7); 3/sex/time point, six time points (each animal will be sampled at three time points; 0.5 mL per sample) Bioanalysis Toxicokinetic analysis
Terminal observations	<ul style="list-style-type: none"> MTD: Necropsy/Macroscopic examination at termination of observation period; no tissue retention Repeat dose: Necropsy/Macroscopic examination at termination of dosing; tissue retention for possible histopathology/microscopic examination (optional)

TABLE 2.3**A MTD/Repeat-Dose Range-Finding Toxicity Study in Nonrodents (Non-GLP)**

Duration	<ul style="list-style-type: none"> Up to 3 weeks
Species /age at treatment initiation	<ul style="list-style-type: none"> Beagle dogs, Cynomolgus monkeys, or Minipigs
Age at treatment initiation	<ul style="list-style-type: none"> Dependent on species
Animals	<ul style="list-style-type: none"> Six (three males and three females) Two for MTD study; four for range-finding study
Dose Procedure	<ul style="list-style-type: none"> MTD: The test article will be administered to one male and one female in up to four single doses, each followed by a 3- or 4-day post-dose observation and drug washout period. The dose will be increased or decreased for each cycle, based on responses to preceding doses, until dose-limiting toxicity is observed (MTD), after which no additional doses will be administered. If no MTD is established after four dose cycles, the Sponsor will be consulted for a decision on whether additional doses should be administered. Repeat dose: After the MTD has been established, the MTD or another dose selected in consultation with the Sponsor will be administered to the previously untreated animals (2/sex) for 7 consecutive days.
Observations	<ul style="list-style-type: none"> Clinical signs: daily Physical examinations: MTD: prior to each dose, 3–4 days following the final dose. Repeat dose: pretest and termination. Body weights: MTD: prior to each dose, 3–4 days following final dose. Repeat dose: pretest, Day 3, and termination. Food consumption: daily, beginning at least 1 wk pretest. Hematology, clinical chemistry: pretest and termination—all animals in the repeat-dose phase
Toxicokinetics (optional)	<ul style="list-style-type: none"> Sampling: Days 1 and 7 for the repeat-dose phase; six time points Bioanalysis Toxicokinetic analysis
Terminal observations	<ul style="list-style-type: none"> MTD: None. Animals will be returned to the holding colony or euthanized. Repeat dose: Necropsy/Macroscopic examination at termination of dosing; tissue retention for possible histopathology/microscopic examination (optional)

REGULATORY GUIDELINES: STUDY REQUIREMENTS

LIST OF GUIDELINES AND AGENCIES

Toxicology Testing

General

The most universally accepted general toxicity testing guidelines are those published by the Organization for Economic Cooperation and Development (OECD) and accepted in many countries. These guidelines were developed for international use and are considered to be “adequate for the evaluation of most chemicals.” Initial drafts were issued in 1979 by the lead countries (the United States and the United Kingdom), and they are reviewed and updated periodically.

OECD Guidelines for Acute Subchronic and Chronic Oral and Dermal Toxicity Studies are as follows:

Duration	Guideline No.	Issue Date	Title
Acute	401	2/24/87	Acute Oral Toxicity
	402	2/24/87	Acute Dermal Toxicity
	420	2/8/02	Acute Oral Toxicity—Fixed Dose Method
	423	2/8/02	Acute Oral Toxicity—Acute Toxic Class Method
	425	10/16/08	Acute Oral Toxicity—Up-and-Down Procedure
Subchronic	407	10/16/08	Repeated Dose 28-Day Oral Toxicity Study in Rodents
	408	9/21/98	Repeated Dose 90-day Oral Toxicity Study in Rodents
	409	9/21/98	Repeated Dose 90-day Oral Toxicity Study in Nonrodents
	410	5/12/81	Repeated Dose Dermal Toxicity: 21/28-Day Study
	411	5/12/81	Subchronic Dermal Toxicity: 90-Day Study
Chronic	452	9/8/09	Chronic Toxicity Studies
	453	9/8/09	Combined Chronic Toxicity/ Carcinogenicity Studies

Pharmaceuticals

General (International) Harmonization (ICH)

The ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) has developed several harmonized, universally accepted, guidelines for acute, subchronic, and chronic toxicity testing of pharmaceuticals, which have been established and accepted by regulatory agencies in all member countries:

The United States: The US Food and Drug Administration (FDA) regulates approval of drugs and publishes Guidances for preclinical development of pharmaceuticals. These guidances are consistent with ICH guidelines.

Europe: The European Medicines Agency (EMA) establishes guidelines for preclinical testing of pharmaceuticals,

generally prepared by the Committee for Medicinal Products for Human Use (CHMP), and accepts ICH guidelines.

Japan: JMHLW (Japanese Ministry of Health, Labor and Welfare): Guidelines for Toxicity Studies of Drugs; Notification No. 88 of the Pharmaceutical Affairs Bureau.

Food Additives

FDA Bureau of Foods: Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food, 2003 (The “Redbook”).

Guidelines, presented in Appendix II (Guidelines for Toxicological Testing), for Acute, Subchronic, and Chronic Oral Toxicity Studies are as follows:

Date	Guideline for:
1993	Acute Oral Toxicity Studies
11/03	Short-Term Oral Toxicity Studies in Rodents and Nonrodents
11/03	Subchronic Oral Toxicity Studies in Rodents and Nonrodents
7/07	Chronic Toxicity in Rodents
11/03	One Year Toxicity in Nonrodents

Agricultural and Industrial Chemicals

In August 1998, the United States Environmental Protection Agency developed a series of guidelines for the testing of pesticides and toxic substances. These were developed by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) which has subsequently been named the Office of Chemical Safety and Pollution Prevention (OCSPP). These guidelines, referred to as the OCSPP guidelines, harmonize, update, and supersede previous guidelines developed specifically for agricultural chemicals (FIFRA, Federal Insecticide, Fungicide and Rodenticide Act) and industrial chemicals (TSCA, Toxic Substances Control Act).

Guidelines for Acute, Subchronic and Chronic Oral and Dermal Toxicity Studies are as follows:

Duration	Guideline	Title
Acute	OCSPP 870.1100	Acute Oral Toxicity Study
	OCSPP 870.1200	Acute Dermal Toxicity Study
Subchronic	OCSPP 870.3050	28-Day Oral Toxicity in Rodents
	OCSPP 870.3100	90-Day Oral Toxicity in Rodents
	OCSPP 870.3150	90-Day Oral Toxicity in Nonrodents
	OCSPP 870.3200	21/28-Day Dermal Toxicity
	OCSPP 870.3250	90-Day Dermal Toxicity
Chronic	OCSPP 870.4100	Chronic Toxicity
	OCSPP 870.4300	Combined Chronic Toxicity/ Carcinogenicity

Japan continues to have separate guidelines for agricultural and industrial chemicals:

Agricultural Chemicals

JMAFF (Japanese Ministry of Agriculture, Forestry, and Fisheries) Guidance on Toxicology Study Data for

Application of Agriculture Chemical Registration, 59 Nohsan No. 4200, January 28, 1985.

Industrial Chemicals

METI (Ministry of Economy, Trade, and Industry): Japan Chemical Substances Control Law (CSCL), enacted April 1, 1987; amended April 2011.

Regulatory Guidelines: Good Laboratory Practices

International

OECD Principles of Good Laboratory Practices [ENV/MC/CHEM (98) 17].

Europe

European Economic Commission (EC) Good Laboratory Practice Regulations Directive 2004/10/EC Feb 11, 2004 (Official Journal No. L 50/44).

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

The United States

Food Additives and Pharmaceuticals: Part 58 of 21 CFR (FDA Good Laboratory Practice Regulations).

Agricultural Chemicals: Part 160 of 40 CFR (EPA/FIFRA Good Laboratory Practice Standards).

Industrial Chemicals: Part 792 of 40 CFR (EPA Good Laboratory Practices—TSCA).

Japan

Pharmaceuticals: Japanese Ministry of Health, Labor and Welfare (JMHLW) Good Laboratory Regulations (Ordinance No. 21, March 26, 1997).

Agricultural Chemicals: Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) Good Laboratory Practice Regulations, Notification No. 3850).

SUMMARY/COMPARISON TABLES

(TABLES 2.4 THROUGH 2.17)

Tables 2.4 through 2.16 list parameters suggested/specified by various study guidelines.

Clinical pathology studies, organ weights, and tissues for histopathology currently recommended for pharmaceutical studies in our laboratory are listed in Table 2.17.

Age of Dogs

Based on the difficulty of interpreting observations in reproductive organs in dogs (it is not always clear if a finding is a test article effect or variation in maturity), our laboratory recommends that dogs be sexually mature at study termination/necropsy. To achieve this, recommended ages of dogs at study initiation are as follows:

Study Duration	Age at Initiation of Study
2–4 weeks	9 months or older
3 months	7 months or older
6 months or longer	~6 months

TABLE 2.4

Animal Requirements—Standard Study Guidelines

Subchronic (2-, 3-, or 4-Week, 90-Day)				Chronic		
Species	Oral dermal	Rodent nonrodent	Rat Dog, NHP, minipig ^a	Rodent nonrodent	Rat Dog, NHP, minipig ^a	
Age/weight at initiation of treatment	Oral	Rat	Before 6 weeks (no more than 8 weeks)	Rat	Before 6 weeks (no more than 8 weeks)	
		Dog minipig	4–6 months (no more than 9 months) ^b 4–5 months	Dog	4–6 months (no more than 9 months) ^b 4–5 months	
	Dermal	Rat, minipig, or rabbit ^a	Adult: Rats: 200–300 g Minipigs: 4–5 months Rabbits: 2–3 kg			
	No. of groups	At least four (control plus at least three dose levels) dermal studies require at least five groups, two controls: Vehicle- and sham-treated			At least four (control plus at least three dose levels) dermal studies require five groups, two controls: Vehicle- and sham-treated	
No. of animals per group	Rats	10/sex	Exceptions: 1. OECD: 5/sex for 2-, 3-, and 4-weeks studies 2. Redbook: 20/sex for 90-day studies	Rat	20/sex	Exception: JMHW: 10/sex
	Nonrodent	4/sex	Exception: JMHW: 3/sex	Nonrodent	4/sex	Exception: JMHW: 3/sex

Note: See also designs in Tables 2.2 and 2.3.

^a NHP—Nonhuman primates. The minipig is the preferred nonrodent model for dermal studies.

^b See below for current recommendations for age of dogs.

^c Refinements using fewer animals for acute oral toxicity studies have been developed (OECD guidelines 420, 423, and 425).

TABLE 2.5
Body Weight and Food Consumption Intervals—Standard Study Guidelines

Study Type	Intervals	Exceptions
Body Weights		
Subchronic	Pretest and weekly	
Chronic	Pretest, weekly through 13 weeks, every 4 week (or approximately monthly) thereafter	1. OECD: at approximately 3-month intervals after 13 weeks 2. Redbook: weekly for dogs
Food Consumption^a		
Subchronic	Pretest and weekly	
Chronic	Pretest, weekly through 13 weeks, every 4 weeks (or approximately monthly) thereafter	1. OECD: at approximately 3-month intervals after 13 weeks 2. Redbook: weekly for dogs 3. JMHW: weekly for dietary administration

^a For nonrodents (dogs and minipigs), food consumption is generally measured (or estimated) daily. For nonhuman primates, many laboratories rely on clinical assessment of appetite/food consumption rather than measurements of food consumed.

TABLE 2.6
Ophthalmology Intervals—Standard Study Guidelines
(Subchronic and Chronic Studies)^a

Guideline	Duration					
	2–4 Weeks		3 Months		Chronic	
	Intervals	Groups	Intervals	Groups	Intervals	Groups
OECD	N.S.	N.S.	Pre and Term	C&H	N.S.	N.S.
EC	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JMHW	R—Rx	All	R—Rx	All	R—Rx	All
	NR—Pre and Rx	All	NR—Pre and Rx	All	NR—Pre and Rx	All
Redbook	Pre and Term	C&H	Pre and Term	C&H	Pre, every 3 months	C&H
OCSPP	Pre and Term	C&H	Pre and Term	C&H	Pre and Term	C&H
JMAFF	Pre and Term	C&H	Pre and Term	C&H	Pre and Term	All
METI	N.S.	N.S.	N.A.	N.A.	N.S.	N.S.

^a N.S., not specified; N.A., not applicable (no guideline); R, rodent; NR, nonrodent; Pre, pretest (before initiation of dosing); Term, termination of dosing; C&H, at least control and high-dose groups; All, all groups if findings in high-dose group; Rx, at least once during treatment.

TABLE 2.7
Clinical Pathology Intervals—Standard Study Guidelines (Subchronic and Chronic Studies)^a

Guideline	Duration								
	2–4 Weeks			3 Months			Chronic		
	Intervals	Animals	Studies	Intervals	Animals	Studies	Intervals	No. Animals (per Sex/Group)	Studies
OECD	Term	All	H,C	R: term NR: P, R,T	All	H,C H,C,U	3,6,12,18,24 months (H,U) 6,12,18,24 months (C)	R: 10 NR: all	H,U,C
EC	R: term NR: pre and term	All	H,C,U	R: term NR: pre and term	All	H,C,U	R: term NR: pre and term	All	H,C,U
JMHW	R: term NR: pre and term	All ^b All	H,C,U H,C,U	R: term NR: P, R,T	All ^b All	H,C,U H,C,U	R: H,C: term U: R, NR: H,C: P, R, T U:	All ^b All	H,C,U
Redbook	Term	R: 5/sex N.R: all	H,C	Term	All	H,C	Pre, R NR: pre, every 3 months;	R: 10 NR: all	H,C
OCSPP	Term	All	H,C	R: term NR: P, R, T	All	H,C	Term every 6 months, term (H,U) Pre, R, term (C)	R: 10 NR: all	H,U,C
JMAFF	N.R.	N.R.	N.A.	R: term NR: P, R, T	All	H,C,U ^c	Every 6 months, term	R: 10 NR: all	H,C,U
METI	N.S.	N.S.	H,C	N.A.	N.A.	N.A.	N.S.	N.S.	H,C,U

^a N.S., not specified; N.A., not applicable (no guideline); N.R., not required; R, rodent (rabbits included for dermal studies); NR, nonrodent; P or Pre, pretest (before initiation of dosing); T or term, termination of dosing; R, at least once during treatment; H, hematology; C, clinical chemistry; U, urinalysis.

^b Urinalysis can be performed on a fixed number of animals per group.

^c Urinalysis performed at termination only (if considered necessary).

TABLE 2.8
Hematology Parameters of Subchronic and Chronic Studies—Standard Study Guidelines

Hematology All Guidelines
Erythrocyte count
Hematocrit
Hemoglobin concentration
Leukocyte count (total and differential)
Some measure of clotting function
Suggestions: Clotting time
Platelet count
Prothrombin time (PT)
Activated partial thromboplastin time (APTT)
Exceptions/Additions
OCSPP: MCV, MCH, MCHC
MHW: in addition, reticulocyte count, PT, APTT
JMAFF: specifies platelet count
METI chronic studies: in addition, reticulocyte count; specifies platelet count
EC: Guidelines do not specify parameters

TABLE 2.9
Clinical Chemistry Parameters of Subchronic and Chronic
Studies—Standard Study Guidelines

Parameter	Guidelines ^a					
	OECD	JMHW	RDBK	OCSP	JMAFF	METI
Alkaline phosphatase		x		x	x	x
Alanine aminotransferase (ALT)	x	x	x	x	x	x
Aspartate aminotransferase (AST)	x	x	x	x	x	x
γ -glutamyltransferase (GGT)	x		x	x		x
Glucose	x	x	x	x	x	x
Bilirubin: total	x	x	x	x ^c		
Creatinine	x	x	x	x		x
Urea (BUN)	x	x	x	x	x	x
Total protein	x	x	x	x	x	x
Albumin	x	x	x	x	x	
Albumin/globulin ratio		x				
Electrolytes (Na, K, Cl, Ca, P)	x	x	x	x	x	x
Cholesterol		x		x		x
Triglycerides		x		x		x
Ornithine decarboxylase (ODC)	b	b				b
Protein-electrophoretogram		x				
Lactate dehydrogenase						c
Creatine kinase						c
Phospholipids						c
Uric acid						c

^a EC guidelines do not specify parameters.

^b Ornithine decarboxylase is a tissue enzyme; no acceptable analytical procedure for blood exists.

^c Chronic studies only.

TABLE 2.10
Urinalysis Parameters of Subchronic and Chronic Studies—Standard
Study Guidelines

Parameter	Guideline ^a				
	OECD	MHW	OCSP	JMAFF	METI (Chronic)
Appearance	x		x	x	
Volume	x	x	x	x	x
Specific gravity	x	x	x	x	
Protein	x	x	x	x	x
Glucose	x	x	x	x	x
Ketones		x		x	x
Occult blood ^b	x	x	x	x	x
Sediment microscopy ^b		x	x	x	c
pH	x	x	x		x
Bilirubin		x	x		x
Urobilinogen		x			x
Electrolytes (Na, K, etc.)		x			

^a EC guidelines do not specify parameters; other guidelines do not recommend routine urinalyses.

^b Semiquantitative evaluation.

^c When necessary.

TABLE 2.11
Organ Weight Requirements—Standard Study Guidelines

Organ to be Weighed	Guideline ^a					
	OECD	JMHW	RDBK	OCSPP	JMAFF	METI
Adrenal glands	×	×	×	×	×	×
Kidneys	×	×	×	×	×	×
Liver	×	×	×	×	×	×
Testes	×	×	×	×	×	×
Epididymides	×					
Ovaries	×	×		×		×
Thyroid/parathyroids	NR	^b	NR	NR	NR	
Brain	×	×		×	Chronic	Chronic
Heart	×	×		×		Chronic
Lungs		^b				Chronic
Spleen	×	×		×		Chronic
Pituitary		×				Chronic
Salivary gland		^b				
Seminal vesicles		^b				
Thymus	×	^b		×		
Uterus	×	^b		×		

^a EC guidelines do not specify organs to be weighed. NR, nonrodent.

^b Guidelines state that these organs are “often weighed.”

TABLE 2.12
Microscopic Pathology Requirements of Subchronic and Chronic Studies (Rodents)—General—Standard Study Guidelines

Groups	Control and High-Dose All Tissues ^a	Intermediate Doses					
		Target Organs ^b	Lesions/ Masses	Early Deaths ^c	Lungs ^d	Liver	Kidneys
OECD	×	×	×	C			
JMHW	×	×					
Redbook	×	×	×	× ^e	C	C	C
OCSPP	×	×	×	× ^e			
JMAFF	×	×	×	×	×	×	×
METI	×	×					

Note: Nonrodents: most guidelines recommend examinations for all animals. Exception: OECD 90-day study: control and high-dose, all tissues; intermediate dose, target organs.

^a Tissues listed in Tables 2.13 or 2.15, chronic studies only; SC, subchronic studies only.

^b Target organs: organs for which possible test material-related effects are seen in high-dose animals.

^c Animals found dead or euthanatized in moribund condition before study termination (all tissues).

^d Examination recommended, primarily for evidence of infection.

^e Not required for 21/28-day studies.

^f Not required for dermal studies.

TABLE 2.13**Microscopic Pathology Requirements—Tissues Most Often Recommended for Subchronic Studies—Standard Study Guidelines**

Tissues ^a	OECD		EC	JMHW	RDBK		JMAFF	OCSPP	METI
	28 Day	90 Day			28 Day	90 Day			
Adrenal glands	×	×	×	×	R	×	×	×	×
Bone (sternum/femur/ vertebrae/rib)			S, F, or V	S, F		S	S, F	×	F+
Bone marrow (sternum/ femur/vertebrae/ rib)	×	×	S, F, or V	S, F	R	S	S, F	×	F+
Brain (medulla/pons, cerebrum, cerebellum)	×	×	×	×	R	×	×	×	+
Esophagus		×	×	×	×	×	×	×	
Heart	×	×	×	×	×	×	×	×	×
Kidney	×	×	×	×	×	×	*×	×	×
Large intestine (cecum, colon, rectum)	×	×	Colon	×	R	×	×	×	
Liver	×	×	×	×	×	×	*×	×	×
Lung (with mainstem bronchi)	×	×	×	×	×	×	×	×	+
Lymph node (representative)	×	×	×	×	R	×	×	×	
Ovaries	×	×	×	×	×	×	×	×	+
Pancreas		×	×	×	×	×	×	×	
Pituitary		×	×	×		×	×	×	
Prostate	×	+	×	×	×	×	+		
Salivary glands		+	×	×		×	×	×	
Small intestine (duodenum, ileum, jejunum)	×	×	×	×	R	×	×	×	
Spleen	×	×	×	×	×	×	×	×	×
Stomach	×	×	×	×	R	R	×	×	+
Testes (with epididymides)	×	×	×	×	×	×	×	×	+
Thymus	×	×	×	×		×	×	×	
Thyroid (with parathyroids)	×	×	×	×		×	×	×	+
Trachea	×	×	×	×		×	×	×	
Urinary bladder	×	×	×	×		×	×	×	+
Uterus	×	×	×	×	R	×	×	×	
Gross lesions/masses/ target organs	×	×	×	×	×	×	*×	×	×

^a *, 21/28 day dermal studies; +, examination/preservation required only if indicated by signs of toxicity or target organ involvement (for, METI, preservation suggested, examination not required); R, tissue required for rodents only.

TABLE 2.14**Microscopic Pathology Requirements—Tissues Occasionally Recommended for Subchronic Studies—Standard Study Guidelines**

Tissue ^a	OECD		EC	JMHW	RDBK		JMAFF	METI
	28 Day	90 Day			90 Day	OCSPP		
Aorta		×			×	×	×	
Eyes		+	×	×	×	+		+
Gallbladder (not present in rats)			×	×	×	×	×	
Lacrimal gland (rodent only)				×				
Larynx						×		
Mammary gland		/+	×	×	×	/	/	
Muscle (skeletal, usually biceps femoris)		+			×		×	
Nasopharyngeal tissue						×		
Nerve (peripheral/sciatic)	×	×			×	×	×	
Seminal vesicles (not present in dogs)		+		×	×	×	×	
Skin		(×)		×		(×)	*×	
Spinal cord (no. of sections; total no. indicated)	3	3	×	×	2	3	×	
Tongue			×					
Vagina			×					
Zymbal glands							+	

^a /, From females only; *, 21/28 day dermal studies; +, examination/preservation required only if indicated by signs of toxicity or target organ involvement (for METI, preservation suggested, examination not required); R, tissue required for rodents only; (), dermal only.

TABLE 2.15
Microscopic Pathology Requirements—Standard Study Guidelines—Tissues Most Often Recommended for Chronic Studies

Tissues ^a	OECD	EC	JMHW	RDBK	OCSPP	JMAFF	METI
Adrenal glands	×	×	×	×	×	×	×
Bone (sternum/femur/vertebrae)	S, F	S, F, or V	S, F	S	×	S, F	S, F, or V
Bone marrow (sternum/femur/ vertebrae)	S	S, F, or V	S, F	S	×	S, F	S
Brain (medulla/pons, cerebrum, cerebellum)	×	×	×	×	×	×	×
Esophagus	×	×	×	×	×	×	×
Heart	×	×	×	×	×	×	×
Kidney	×	×	×	×	×	×	×
Large intestine (cecum, colon, rectum)	×	Colon	×	×	×	×	×
Liver	×	×	×	×	×	×	×
Lung (with mainstem bronchi)	×	×	×	×	×	×	×
Lymph node (representative)	×	×	×	×	×	×	×
Mammary gland	/ ^a	×	×	×	/	/	/
Ovaries	×	×	×	×		×	×
Pancreas	×	×	×	×	×	×	×
Pituitary	×	×	×	×	×	×	×
Prostate	×	×	×	×	×	×	×
Salivary glands	×	×	×	×	×	×	×
Small intestine (duodenum, ileum, jejunum)	×	×	×	×	×	×	×
Spleen	×	×	×	×	×	×	×
Stomach	×	×	×	×	×	×	×
Testes (with epididymides)	×	×	×	×	×	×	×
Thymus	×	×	×	×	×	×	×
Thyroid (with parathyroids)	×	×	×	×	×	×	×
Trachea	×	×	×	×	×	×	×
Urinary bladder	×	×	×	×	×	×	×
Uterus	×	×	×	×	×	×	×
Gross lesions/masses/target organs	×	×	×	×	×	×	×

^a /, From females.

TABLE 2.16
Microscopic Pathology Requirements—Tissues Occasionally Recommended for Chronic Studies—Standard Study Guidelines

Tissue	OECD	EC	JMHW	RDBK	JMAFF	OCSPP	METI
Aorta	×			×	×	×	
Eyes	×	×	×	×		×	×
Gallbladder (not present in rats)		×	×	×	×	×	
Lacrimal gland (rodent only)			×				
Larynx							×
Fallopian tubes					×		
Muscle (skeletal, usually biceps femoris)	×			×	×		
Nerve (peripheral/sciatic)	×			×	×	×	×
Nose					×		
Pharynx					×		
Seminal vesicles (not present in dogs)	×		×	×	×	×	×
Skin	×		×		×	×	×
Smooth muscle			×				
Spinal cord (number of sections; total number indicated)	3	×	×	2	×	3	×
Tongue			×				×
Vagina			×				

TABLE 2.17

Suggested Clinical Pathology, Organ Weights, and Tissues for Histopathology—Pharmaceutical Studies

1. Clinical Pathology (all Standard Species Except as Noted)

A. Hematology									
Hematocrit (Hct)	Red blood cell count (RBC)	Hemoglobin concentration (Hgb)	Mean corpuscular hemoglobin concentration (MCHC)	Mean corpuscular hemoglobin (MCH)	Mean corpuscular volume (MCV)				
Red cell distribution width (RDW)	Absolute reticulocyte count (Retic)	Total white blood cells + absolute differential counts	Platelet count (Plt)	Peripheral blood smear (collected)	Bone marrow smear (collected; not read)				
B. Coagulation (not in mice)									
Activated partial thromboplastin time (aPTT; terminal only for rodents)		Prothrombin time (PT; terminal only for rodents)				Fibrinogen (Fib; nonrodents only)			
C. Clinical chemistry									
Aspartate aminotransferase (AST)	Alanine aminotransferase (ALT)	Alkaline phosphatase (ALP)	Gamma-glutamyl transferase (GGT) (nonrodents only)	Total bilirubin (tBili)					
Total protein (TP)	Albumin (Alb)	Globulin (calculated) (Glob)	Albumin/globulin ratio (calculated) (A/G)	Cholesterol (enzymatic) (Chol)					
Triglycerides (Trig)	Glucose (Glu)	Urea Nitrogen (UN)	Creatinine (Creat)	Total calcium (tCa)					
Phosphorus (P)	Sodium (Na)	Potassium (K)	Chloride (Cl)	GLDH (minipig only)					
D. Urinalysis (not in mice)									
Urine volume		Specific gravity (USG)		Appearance (color, clarity)					
Qualitative chemistry (dipstick): pH, proteins, ketones, bilirubin, glucose, urobilinogen, occult blood.									
If dipstick protein ≥ 100 mg/dL (+2) \rightarrow evaluation of urine sediment.									

2. Organ Weights

Species	All Species Except Mouse + Dog		Mouse	Dog
Adrenals	+		–	+
Brain	+		+	+
Epididymides	+		+	+
Heart	+		+	+
Kidneys	+		+	+
Liver	+		+	+
Lungs (for inhalation studies only)	(+)		(+)	(+)
Prostate (a)	+		+	+
Seminal vesicles (a)	+		–	–
Spleen	+		+	+
Testes	+		+	+
Thymus	+		+	+
Pituitary, thyroid/parathyroid	+		–	+
Ovaries	+		+	+
Uterus	+		+	+

Note: (a), Rabbit and rat: prostate and seminal vesicle are weighed together.

(continued)

TABLE 2.17 (continued)
Suggested Clinical Pathology, Organ Weights, and Tissues for Histopathology—Pharmaceutical Studies

3. Tissues for Histopathology

Adrenal glands	Harderian gland (rodent only)	Pancreas	Spleen
Aorta	Heart	Parathyroid gland	Stomach
Bone/bone marrow (distal femur w/ articular cartilage, sternum); + BM smear	Ileum	Peripheral nerve (sciatic)	Testes
Brain	Jejunum	Peyer's patches/GALT	Thymus
Cecum	Kidney	Pituitary	Thyroid
Colon	Lacrimal gland	Prostate	Trachea
Duodenum	Liver	Salivary gland (submandibular)	Urinary bladder
Epididymis	Lung w/ bronchi	Seminal vesicle (not in dog)	Uterus + Vagina
Esophagus	Lymph node (mesenteric, axillary)	Skeletal muscle (<i>rectus femoris</i>)	Gross lesions/abnormalities
Eye	Mammary gland (F, all species; M, rat)	Skin (dorsal—base of tail)	Route of administration (see following tables) ^a
Gallbladder (not in rat)	Ovary	Spinal cord (C, T and L)	Masses (w/ regional lymph nodes)

Note: Optic nerve, rectum, tongue, and zymbal gland: collected but not examined histopathologically. Mammary gland in male and GALT evaluated only if present on routine sections.

^a Inhalation/nasal studies → nasal turbinates, larynx, nasopharynx/pharynx, tracheobronchial lymph nodes, tracheal bifurcation w/ carina. Injection/infusion studies → injection/infusion sites (skin, muscle, vessels). Dermal/SQ/IM studies → all sites. For injection/topical sites → add draining lymph nodes.

STUDY IMPLEMENTATION

PROTOCOL COMPONENTS CHECKLIST

This is a form that has been used in our laboratory to outline protocol components for toxicity studies.

Protocol Request

Sponsor: _____
 Address: _____
 Attention: _____
 Agent: _____
 Industry: _____

Date of Contact: _____
 Contact Recipient: _____
 Program (Y/N): _____
 Information _____

Study Type

Toxicity

- ☐ MTD/Range-Finding
☐ Toxicity
☐ Carcinogenicity
☐ _____

Duration of Study

☐ _____ Hours ☐ _____ Days ☐ _____ Weeks ☐ _____ Months ☐ _____ Years

Species/Strain

☐ Rat/_____ ☐ Minipig ☐ Swine ☐ Mouse/_____ ☐ Rabbit ☐ Monkey ☐ Others/_____

Route of Administration

Oral

☐ Diet ☐ Adj. ☐ Const.
☐ Gavage ☐ Capsule
☐ _____
☐ _____

Inhalation

☐ Head-Only
☐ Nose-Only
☐ Whole-Body
☐ _____

Dermal

☐ Open
☐ Semi-Occluded
☐ Occluded
☐ _____

Injection/Infusion

☐ SubQ ☐ IP ☐ IM ☐ IV
☐ Infusion (Continuous)
☐ Infusion (Intermittent)
☐ Bolus Injection

Others

☐ Vaginal
☐ Nasal
☐ Ocular _____

Frequency of Administration

☐ Single ☐ Daily #: _____ ☐ Weekly #: _____ ☐ _____ h/ _____ days/week ☐ Others

Regulatory Guideline

☐ OCSPP ☐ FDA/Pharmaceutical ☐ OECD ☐ JMETI
☐ FDA/Biologic ☐ JMAFF ☐ EC ☐ WHO
☐ FDA/Red Book ☐ JMHV ☐ Nonregulatory ☐ Others _____

Experimental Design

Group	I		II		III		IV		V		VI	
	M	F	M	F	M	F	M	F	M	F	M	F
Dosage <input type="checkbox"/> ppm <input type="checkbox"/> mg/kg <input type="checkbox"/> mL/kg <input type="checkbox"/> mg/M ³ <input type="checkbox"/> mL(cc) <input type="checkbox"/> _____												
Number of Animals (Main Study)												
<input type="checkbox"/> Satellite/Recovery												
Ophthalmology												
<input type="checkbox"/> Intervals: Main Study												
Satellite/Recovery												
Hematology												
<input type="checkbox"/> Intervals: Main Study												
Satellite/Recovery												
<input type="checkbox"/> Standard Battery <input type="checkbox"/> Additional (see attached)												
Clinical Chemistry												
<input type="checkbox"/> Intervals: Main Study												
Satellite/Recovery												
<input type="checkbox"/> Standard Battery <input type="checkbox"/> Additional (see attached)												
Urinalysis												
<input type="checkbox"/> Intervals: Main Study												
Satellite/Recovery												
<input type="checkbox"/> Standard Battery <input type="checkbox"/> Additional (see attached)												
Group	I		II		III		IV		V		VI	
	M	F	M	F	M	F	M	F	M	F	M	F
Toxico/Pharmacokinetics												
<input type="checkbox"/> Intervals: Main Study												
<input type="checkbox"/> Timepoints: Satellite/Recovery												
<input type="checkbox"/> Plasma <input type="checkbox"/> Serum <input type="checkbox"/> In-House Analysis <input type="checkbox"/> Shipped to Sponsor												
ECG												
<input type="checkbox"/> Intervals: Main Study												
Satellite/Recovery												
Blood Pressure												
<input type="checkbox"/> Intervals: Main Study												
Satellite/Recovery												
Necropsy												
<input type="checkbox"/> Interim Sacrifice												
<input type="checkbox"/> Terminal Sacrifice												
<input type="checkbox"/> Recovery Sacrifice												
<input type="checkbox"/> Organ Weights												
<input type="checkbox"/> Microscopic Examination												
Analytical												
<input type="checkbox"/> Dose Medium <input type="checkbox"/> Product Chemistry <input type="checkbox"/> RIA <input type="checkbox"/> ELISA												
Others												
<input type="checkbox"/> Special Stains <input type="checkbox"/> Special Statistical Package Status Reports: <input type="checkbox"/> Weekly <input type="checkbox"/> Monthly <input type="checkbox"/> Interim Only <input type="checkbox"/> Term Only												

STUDY INITIATION CHECKLIST

The following checklist presents activities to be completed plus items to be addressed before initiation of the study.

1. Protocol		
a. Finalized/scientific review and approval		_____
b. IACUC review and approval		_____
c. QA GLP review and approval		_____
d. Distributed to appropriate personnel		_____
2. Test Animals		
a. Room assigned		_____
b. Animals ordered		_____
c. Animals received		_____
d. Health examination completed (including prestudy ECG, ophthalmology, vaccinations, TB testing as required)		_____
e. Assigned to groups; assignment reviewed and approved by Study Director		_____
3. Prestudy Clinical Pathology (if required)		
a. Scheduled		_____
b. Completed		_____
c. Reviewed by Study Director (animal selection)		_____
4. Study Schedule		
a. Completed/approved by Study Director		_____
b. Distributed		_____
5. Test/Control Material		
a. Amount needed calculated/confirmed		_____
b. Received		_____
c. Storage _____		_____
d. Handling instructions (personnel protection)		
1) Technical material		_____
2) Dosing solutions, treated feed, etc.		_____
6. Prestudy Analysis of Dose Formulations/ Diets (if required)		
a. Method provided		_____
b. Method validated		_____
c. Scheduled		_____
d. Completed		_____
e. Acceptable		_____
7. Doses, Dose Solutions, Diet Preparation		
A. Doses/concentrations selected		_____
B. Mixing instructions		
a. Developed/provided to appropriate personnel		_____
b. Prestudy mix completed		_____
c. Analysis required/completed/acceptable		_____
Type: Homogeneity	Required	Completed/Acceptable
Stability	_____	_____
Concentration	_____	_____

(continued)

(continued)

8. Waste Disposal	
a. Technical material	_____
b. Dosing solutions, treated feed, etc.	_____
c. Procedures to minimize waste	_____
9. Expected Toxicity and Clinical Signs	
10. Subcontractors/PI's (including pathology, clinical pathology, metabolism, microbiology)	
a. Required	_____
b. QA notified	_____
If required, list below subcontractor/PI's name and address, work to be performed, handling instructions, and the person responsible for shipment:	
11. Necropsy Procedures (special instructions, e.g., photos of gross lesions, paired organs weighed together or separately, unusual fixative, flash-frozen samples)	
12. Toxicokinetic Samples (blood, urine, etc.)	
a. Type	_____
b. Volume needed	_____
c. Preservative/anticoagulant, etc.	_____
d. Storage/transfer instructions	_____
13. Report Schedule	
a. Status reports—frequency	
Weekly	_____
Monthly	_____
b. Special data handling/statistics	_____
c. Final draft—date required	_____
d. Histopathology—date required	_____
e. Final report—date required	_____

GLP PROTOCOL REVIEW CHECKLIST

This is a checklist used by Huntingdon Life Sciences' Quality Assurance Unit to review protocols for GLP Compliance.

Sponsor Code and Study Number:

Auditor:

Date:

	FDA	EPA	OECD	MHLW	MAFF	METI	NON-REG
A descriptive title and statement of purpose of the study							
Identification of the test and control article by name and/or code number							
The name and address of the test facility and the sponsor							
Proposed experimental start date	NA			NA			
Proposed experimental termination date	NA			NA	NA		
Proposed duration of study	NA	NA	NA	NA			
Description of experimental design, including method for control of bias							
Dose levels in appropriate units, and method and frequency of administration							
Method degree of absorption is measured	NA	NA	NA	NA			
Route of administration							
Reason for route	NA						
Reason for frequency and duration	NA	NA	NA				
Justification for selection of test system	NA						
Test, control, and reference substances or mixtures appropriately tested (or to be tested) for identity, strength, purity, stability, uniformity, and solubility							
Where applicable, the number, body weight range, sex, source of supply, species, strain, substrain, and age of the test system							
Environmental conditions for the test system	NA	NA	NA				
Description and/or identification of diet, to include statement of contaminants			NA				
Procedure for unique ID of the test system			NA				
Type/frequency of test measurements							
Records to be maintained							
Date sponsor approved, and dated study director signature					NA		
Signed by test facility management	NA	NA	NA				
Proposed statistical methods			NA				
Reference to OECD or other test guidelines	NA	NA		NA	NA	NA	NA

Key	
√ = Requirement met	√/O = Space provided/to be completed
O = Requirement not met	NA = not applicable

TEST AND/OR CONTROL MATERIAL HANDLING INFORMATION

This is a form used in our laboratory to obtain information about materials to be tested.

Test and/or Control Material
Handling Information

Study No(s):

This form is designed to specify test material handling and disposition instructions and to provide procedures (if known) in case of accidental exposure to the substance. As with all data, this information will be considered confidential and will

be available only to persons involved with the study or studies using this substance. Please provide as complete information as possible for each category below and/or attach the information in your own form (MSDS or COA) and return:

I. Identification

Test Material (Name or Code Number) _____

Batch or Lot Number: _____

Physical Description: _____

Purity: _____

Density (if known): _____

pH (if applicable): _____

Test Material is Soluble in (check one): ____ Water; ____ Acetone; ____ Alcohol; ____ Oil; ____ Other (____)

II. Storage Information Material should be stored in:

____ Temperature-monitored room (60°F–85°F); ____ Freezer; ____ Refrigerator; ____ Other (____)

III. Stability

Length of time material is stable under conditions described above: _____

Expiration date: _____ Indicated on label: Yes/No (circle one)

	Unknown	Less than 4 h	Up to 4 h	Up to 24 h	Other ()
Stability in common vehicles					
Water:	_____	_____	_____	_____	_____
Methylcellulose	_____	_____	_____	_____	_____
Corn oil	_____	_____	_____	_____	_____
Organic solvents (acetone, ethanol)	_____	_____	_____	_____	_____
Other (_____)	_____	_____	_____	_____	_____

If stability of neat material or of material in vehicle to be used in study is not known:

_____ Instructions for analysis will be provided.

_____ Samples are to be submitted to Sponsor for analysis.

_____ Analysis will not be required.

IV. Handling (Employee Safety) Information

Known Hazards: (If available, attach summary of pertinent results of any previous toxicity studies).

Approximate rodent oral LD₅₀ = ____ mg/kg (if unavailable, enter NK, not known).

Is material a probable eye/skin irritant: Yes/No (circle one).

Other Pertinent Information: _____

Precautions: Use of protective clothing (laboratory coats), latex gloves, safety glasses, and dust mask is routine. Precautions in excess of the above should be specified:

_____ Routine precautions adequate. Other: _____

In case of emergency related to this substance, contact:

_____ of _____ at _____

(Person) (Company) (Phone Number)

V. Disposition

All material will be returned to the Sponsor. Person and address to whom samples are to be returned:

Name: _____ Shipping Instructions: _____

Address: _____

Note: Please enclose appropriate shipping labels for return.

VI. Signature

Information submitted by: _____

Company: _____

Date: _____

FLOWCHART/TIME LINES FOR CONDUCT OF STUDY (FIGURES 2.1 AND 2.2)

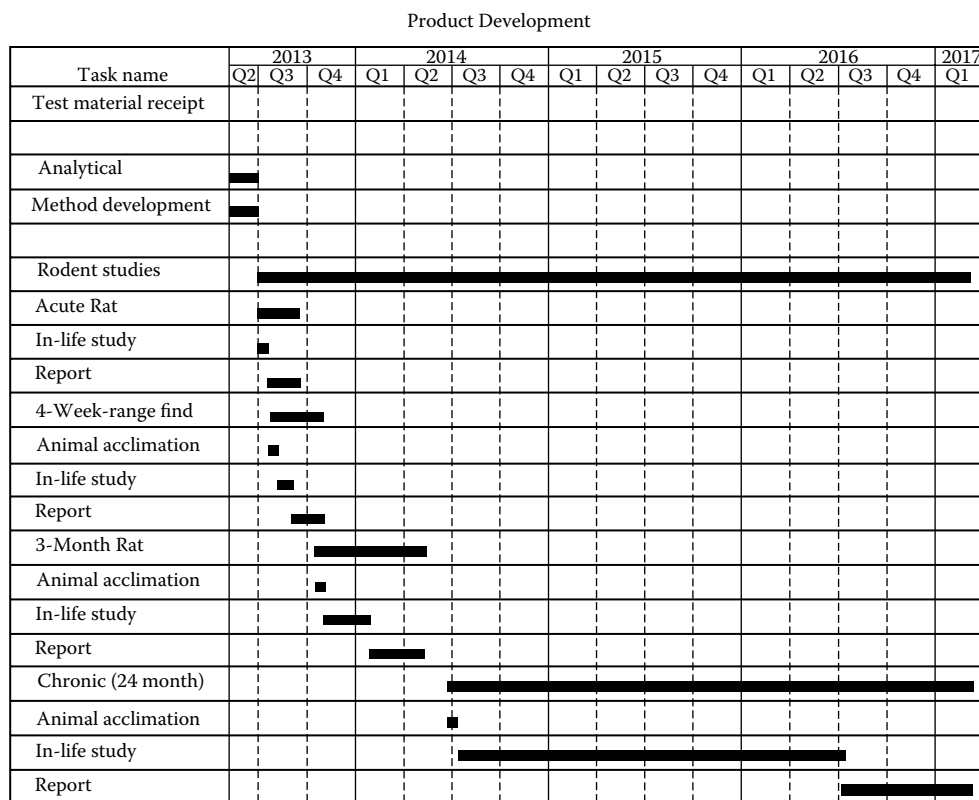


FIGURE 2.1 Flowchart/time line for rodent studies.

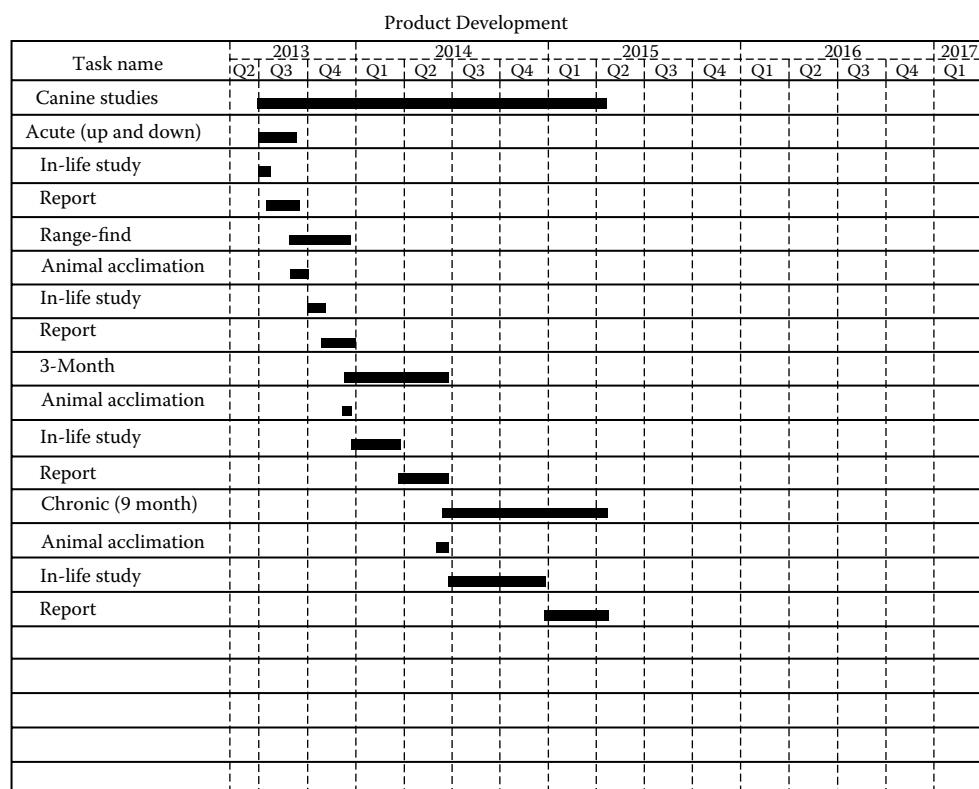


FIGURE 2.2 Flowchart/time line for nonrodent studies.

SAMPLE STUDY SCHEDULE

Proposed Study Schedule Example

Study Number: _____

Page __1__ of __1__

Prepared by: _____ Date: _____

Approved by: _____ Date: _____

Verified by: _____ Date: _____

Scheduled by: _____ Date: _____

cc: _____

Year 2013 Date	Study		Evaluations	B W	F I	F O	O B S	Year 2013 Date	Study		Evaluations	B W	F I	F O	O B S
	Day	Week							Day	Week					
7/1								8/1	14			u		u	u
7/2								8/2	15	3			W		
7/3								8/3	16						
7/4								8/4	17						
7/5	-13	-1	Animal Receipt		N			8/5	18						
7/6	-12							8/6	19						
7/7	-11							8/7	20						
7/8	-10							8/8	21			u		u	u
7/9	-9							8/9	22	4			W		
7/10	-8							8/10	23						
7/11	-7		Vet. Examination	u			u	8/11	24						
7/12	-6	0			W			8/12	25						
7/13	-5							8/13	26						
7/14	-4							8/14	27						
7/15	-3		Ophthalmology					8/15	28			u		u	u
7/16	-2							8/16	29	5	Ophthalmology, Urine				
7/17	-1							8/17	30		Hematology, Necropsy				
7/18	0		Sort	u		u	u	8/18							
7/19	1	1	Initiate treatment		W			8/19							
7/20	2							8/20							
7/21	3							8/21							
7/22	4							8/22							
7/23	5							8/23							
7/24	6							8/24							
7/25	7			u		u	u	8/25							
7/26	8	2			W			8/26							
7/27	9							8/27							
7/28	10							8/28							
7/29	11							8/29							
7/30	12							8/30							
7/31	13							8/31							

BW–Body Weight

FI–Feeder in

W–Weighed Feeder

N–NonWeighed Feeder

FO–Feeder Out

OBS–OBSERVATIONS

General Notes:

ANIMAL SELECTION: RANDOMIZATION PROCEDURES

Rodents are generally assigned to studies by use of computer programs which sort animals by weight and assign them to groups in a manner that results in similar mean weights.

Similar programs can be used for nonrodents (dogs, mini-pigs, or monkeys). The following procedures have also been used in our laboratory to assign small numbers of animals to groups and may produce better group assignment:

1. Eliminate from selection any animals considered unsuitable on the basis of pretest examinations and evaluations.
2. List animals in ascending or descending order based on body weights.
3. Determine the number of blocks per sex and the number of animals per block by referring to the protocol. The number of blocks per sex is equal to the number of groups, and the number of animals per block is equal to the number of animals per group. For example, if the study design calls for four groups of five animals per sex, then the number of blocks per sex equals four with five animals in a block.
4. Distribute the animals into the blocks so that the body weight means for each block are comparable.
5. Do not place littermates (dogs) in the same blocks.
6. Use a random number table to assign the blocks to study groups as follows:
 - a. Assign a two-digit number to each block (00–99).
 - b. When an assigned number is reached on the table, assign this block to Group I.
 - c. Continue across the rows until the next assigned number is reached. If the block assigned to this number is a different sex than the first number found, assign this block as Group I. If it is the same sex as the first block assigned, assign this block to Group II.
 - d. Continue in this manner until all the blocks are assigned to dose groups.

DOSE/VEHICLE SELECTION AND DOSE FORMULATION

DEFINITIONS RELATIVE TO DOSES/DOSE SELECTION

Acute Studies

MTD: The maximum tolerated dose (highest dose that can be tolerated without producing severe toxicity or lethality).

MFD: Maximum feasible dose (often used for dermally and intranasally administered materials, for which the volume that can be administered is limited): The highest dose volume that can be administered at the highest attainable concentration of test article in the dose formulation.

General Studies

Limit Dose: A dose which is considered high enough that if no mortality or significant toxicity is seen in animals receiving this dose, no higher doses are required.

Examples:

Limit Dose (FDA M-3 R-2 Guidance)	1000 mg/kg/day (with a few exceptions)
Limit Doses (EPA/OECD)	
Acute Oral Toxicity	2000 mg/kg
Acute Dermal Toxicity	2000 mg/kg
21-Day Dermal Toxicity	1000 mg/kg/day
Chronic Studies of Pesticides	1000 mg/kg/day

Note: The “limit” dose of a nonnutritive material added to the diet is generally considered to be 5% (50,000 ppm).

Subchronic and Chronic Studies

NOEL: No observed effect level. Dose at which no effect is seen.

NOAEL: No observed adverse effect level. Dose at which no adverse (toxic) effect is seen.

ADI: Acceptable daily intake (established for food additives/residues and published by the EPA).

Chronic Studies

MTD: Maximum tolerated dose. Highest dose that can be tolerated without significant lethality from causes other than tumors. (A frequently cited criterion for EPA studies is that the MTD for chronic studies with pesticides is a dose which produces an approximate 10%–15% decrement in body weight gain.)

GEOMETRIC PROGRESSION TABLES FOR DOSE SELECTION

Table 2.18 is a series of approximately geometrically spaced doses that has been used in our laboratory.

TABLE 2.18
Geometric Progressions

Interval	0.1 log	0.14 log	0.3 log
Multiples of doses	1.26	1.43	2
		0.4 or 0.6	0.25 0.25
		0.8	0.35 0.5
	0.8	1.0	0.5 1.0
	1.0	1.2	0.7 2.0
	1.3	1.7	1.0 4.0
	1.6	2.5	1.4 8.0
	2.0	3.5	2.0 16.0
	2.5	5.0	2.8
	3.2	7.1	4.0
	4.0	10.0	5.6
	5.0	14.2	8.0
	6.3	20.0	11.2
	8.0	28.4	16.0
		40.3	22.4

VEHICLES: GUIDELINES FOR DOSING

The following are commonly used vehicles which are generally regarded as nontoxic and nonirritating.

Oral

Water
Methylcellulose or carboxymethylcellulose (0.5%–5% aqueous suspension)
Oil (corn, peanut, sesame)

Dermal

Physiological saline
Water
Ethanol
Acetone
Mineral oil

Parenteral

Physiological saline (sterile)
Water for injection (sterile)
Additional guidance can be obtained from Gad et al.² and other literature references.

Note: High volumes may be associated with soft stool

**DOSE VOLUME, RATE, AND NEEDLE-SIZE
GUIDELINES (TABLES 2.19 THROUGH 2.21)**

TABLE 2.19
Suggested Dose Volumes (mL/kg) for Test Material Administration

Species	Route													
	Gavage		Dermal		IV		IP		SC ^a		IM ^a		NASAL ^b	
	Ideal	Limit	Ideal	Limit	Ideal	Limit	Ideal	Limit	Ideal	Limit	Ideal	Limit	Ideal	Limit
Mouse	10	20–50	—	—	5	15–25	5–10	25	1–5	10–20	0.1	0.5–1	—	—
Rat	10	20–50	2	—	1–5	10–20	5–10	25	1	10–20	0.1	1	0.1	0.2
Rabbit	10	10–20	2	—	1–3	5–10	—	—	1–2.5	5–10	0.1	1	0.2	1
Dog	10	10–20	—	—	1	5–10	1–3	5	0.5	1–2	0.1	1	0.2	2
Monkey	10	10	—	—	1	5–10	1–3	5	0.5	1–2	0.1	1	0.2	1
Minipig	10	15	0.5	2	1	5–10	1	20	1	2	0.1	0.5	—	—

Source: Adapted from *SYNAPSE*, American Society of Laboratory Animal Practitioners, Vol. 24, March 1991. Some adaptations have been made based on experience in our laboratory. Note that there are many guidelines for this, both published and internal (laboratory-specific) and there is no universal agreement on specific numbers. Recommended dose volumes vary from publication to publication and laboratory to laboratory.

^a Dose per site.

^b Nasal doses (mL/animal, 1/2 dose per nostril) based on experience in our laboratory.

TABLE 2.20
Suggested Dosing Apparatus/Needle Sizes (Gauge) for Test Material Administration^a

Species	Route									
	Gavage		IV		IP		SC		IM	
	Recommended		Ideal	Range	Ideal	Range	Ideal	Range	Ideal	Range
Mouse	French rubber catheter—flexible, 18–20 gauge, 30 mm, bulb-shaped tip		25 or 27	25–30	25 or 27	22–30	25 or 27	22–30	25 or 27	22–30
Rat	French rubber catheter, size 8 or 10		25	25–30	25	22–30	25	25–30	25	22–30
Rabbit	No. 18 French catheter, cut to 15 in., marked at 12 in.		21	21–22	21	18–23	25	22–25	25	22–30
Dog	24–32 French rectal tube—~20 in. long		21	21–22	—	—	22	20–23	21 or 25	20–25
Monkey	14–18 French rectal tube		25	25–30	—	—	22	22–25	25	22–25

^a Recommended gavage equipment and ideal needle sizes are those used in our laboratory. Suggested ranges of needle sizes are from: *Laboratory Manual for Basic Biotechnology of Laboratory Animals*, MTM Associates, Inc.⁴

TABLE 2.21
Guidelines for Dose Administration for Intravenous Infusion Studies

		Species			
		Dog	Primate	Rat	Rabbit
Recommended IV volume (taken from EFPIA guidelines)	IV Bolus (mL/kg)	2.5	2	5	2
	IV Slow injection (mL/kg— max vol)	5	Similar to dog	20	10
	Continuous IV infusion (mL/kg/h)	1	2.5	2.5	1
Surgery not required bolus or intermittent IV infusion (<4 h)		28 or fewer doses (regardless of 1×/day, 2×/day, 1×/week)	14 or fewer doses (regardless of 1×/day, 2×/day, 1×/week)	14 or fewer doses AND fewer than 50 animals	8 or fewer doses
Surgery required	Duration of dose	Over 4 h to continuous	Over 4 h to continuous	Over 4 h to continuous	Over 4 h to continuous
	Number of doses	More than 28 doses	More than 14 doses	More than 14 doses OR over 50 animals	More than 8 doses
VAP/rodent intermittent infusion model		Repeat intermittent infusion dosing for 24 h or less	Repeat intermittent infusion dosing for 24 h or less	2 or fewer doses per week	Repeat intermittent infusion dosing for 24 h or less
Permanent exteriorized catheter		Continuous dosing for over 24 h	Continuous dosing for over 24 h	More than 2 doses per week	Continuous dosing for over 24 h

Note: EFPIA, European Federation of Pharmaceutical Institutes and Associations.

BODY WEIGHT/SURFACE AREA CONVERSION

TABLES (TABLES 2.22 AND 2.23)

TABLE 2.22
Body Weight: Surface Area Conversion Table

Species	Representative Body Weight to Surface Area ^a		
	Body Weight (kg)	Surface Area (m ²)	Conversion Factor (km)
Mouse	0.02	0.0066	3
Rat	0.15	0.025	5.9
Monkey	3	0.24	12
Dog	8	0.4	20
Human			
Child	20	0.8	25
Adult	60	1.6	37

Source: Adapted from Freireich, E.J. et al., *Cancer Chemother. Rep.*, 50, 219, 1966.

^a Example: To express a mg/kg dose in any given species as the equivalent mg/m² dose, multiply the dose by the appropriate km. In human adults, 100 mg/kg is equivalent to 100 mg/kg × 37 kg/m² = 3700 mg/m².

TABLE 2.23
Equivalent Surface Area Dosage Conversion Factors^a

From	To				
	Mouse (20 g)	Rat (150 g)	Monkey (3 kg)	Dog (8 kg)	Human (60 kg)
Mouse	1	1/2	1/4	1/6	1/12
Rat	2	1	1/2	1/3	1/6
Monkey	4	2	1	3/5	1/3
Dog	6	4	3/2	1	1/2
Man	12	7	3	2	1

Source: Adapted from Freireich, E.J. et al., *Cancer Chemother. Rep.*, 50, 219, 1966.

^a This table gives approximate factors for converting doses expressed in terms of mg/kg from one species to an equivalent surface area dose expressed as mg/kg in the other species tabulated.

Example: To convert a dose of 50 mg/kg in the mouse to an equivalent dose in the monkey, assuming equivalency on the basis of mg/m², multiply 50 mg/kg × 1/4 = 13 mg/kg.

DOSE CALCULATIONS: ORAL, DERMAL, OR PARENTERAL ADMINISTRATION

Abbreviations: mL = milliliter; mg = milligram; g = gram (equal to 1000 mg); kg = kilogram (equal to 1000 g); b.w. = body weight (generally expressed in kilograms).

Dose Volume

To calculate dose volume when dose and concentration are known:

$$\text{Dose volume (mL/kg b.w.)} = \frac{\text{Dose (mg/kg b.w.)}}{\text{Concentration (mg/mL)}}$$

Example: To determine the dose volume needed to administer 200 mg of test material per kilogram of body weight of a dose solution containing 20 mg of test material per milliliter:

$$\frac{\text{Dose : 200 mg/kg b.w.}}{\text{Concentration : 20 mg/mL}} = \text{Dose volume : 10 mL/kg b.w.}$$

Concentration of Dosing Mixture

To calculate dose concentration when dose and desired dose volume are known:

$$\text{Concentration (mg/mL)} = \frac{\text{Dose (mg/kg b.w.)}}{\text{Dose volume (mL/kg b.w.)}}$$

Example: To determine the concentration of dose solution needed to administer 200 mg of a test material per kilogram of body weight at a dose volume of 10 mL/kg of body weight:

$$\frac{\text{Dose : 200 mg/kg}}{\text{Dose volume : 10 mL/kg}} = \text{Concentration : 20 mg/mL}$$

Individual Animal Doses

Solids

To calculate the weight of a solid material to be administered to an animal (used for oral administration in capsules, dermal dosing of neat powders): Dose for animal (mg) = dose (mg/kg b.w.) × animal's body weight (kg).

Example: To administer a dose of 25 mg/kg b.w. to a dog weighing 9.4 kg: 25 mg/kg b.w. × 9.4 kg b.w. = 235 mg.

Liquids

To calculate the dose volume to be administered to an individual animal: Dose volume for animal (mL) = dose volume (mL/kg b.w.) × animal's body weight (kg).

Example: To administer a dose of 6 mL of test material per kilogram of body weight to a rat weighing 325 g:

Convert body weight to kilograms: 325 g × 0.001 kg/g = 0.325 kg

Calculate dose: 6 mL/kg b.w. × 0.325 kg b.w. = 1.95 mL

Adjustment for Active Ingredient

If doses are to be administered as doses of pure material or active ingredient, the dose must be adjusted as follows: Desired dose of active ingredient × (100%/active ingredient) = calculated total dose.

Example: To administer a dose of 10 mg/kg b.w. of active ingredient of a test material, which is 85% active:

$$10 \text{ mg/kg} \times \frac{100}{85} = 11.76 \text{ mg/kg (dose to be administered)}$$

Dose Solution Conversions

To convert percent to milligrams per milliliter: Concentration in % × 10 = concentration in mg/mL.

Rationale: A 100% mixture contains 1 g (or 1000 mg)/mL. Therefore,

$$100\% = 1000 \text{ mg/mL}$$

$$10\% = 100 \text{ mg/mL}$$

$$1\% = 10 \text{ mg/mL}$$

Example: To determine the concentration in milligrams per milliliter of a 30% w/v (weight/volume) solution:

$$30\% \text{ w/v} \times 100 = 300 \text{ mg/mL}$$

CALCULATIONS FOR DIETARY AND DRINKING WATER ADMINISTRATION

Abbreviations: t.m. = test material; mg = milligram; g = gram (equal to 1000 mg); kg = kilogram (equal to 1000 g); b.w. = body weight (generally expressed in kilograms); f.c. = food consumption.

Calculation of Concentration: General

- Conversions

Concentrations are usually expressed as ppm (parts per million) or %. Conversion: $\% \times 10,000 = \text{ppm}$.
For solids (dietary administration)

1 ppm = 1 mg of test material per kg of mixture

1% = 10,000 mg of test material per kg of mixture

For liquids (drinking water administration)

1 ppm = 1 mg of test material per liter of mixture

1% = 10,000 mg of test material per liter of mixture

- Calculation of amount of test material needed

To calculate the amount of test material needed when desired concentration and total amount of diet are known:

Desired concentration (ppm) \times required amount of diet (kg) = amount of test material needed (mg).

Example: To prepare 20 kg of a diet containing 6 ppm of test material:

$$6 \text{ ppm} = 6 \text{ mg/kg}$$

$$6 \text{ mg/kg} \times 20 \text{ kg} = 120 \text{ mg}$$

Therefore, mix 120 mg of test material in 20 kg of diet.

- Adjustment for active ingredient

If doses are to be administered as doses of pure material or active ingredient, the concentration must be adjusted as follows: Desired concentration of active ingredient \times (100%/ % active ingredient) = calculated concentration.

Example: To prepare 20 kg of diet containing 6 ppm of active ingredient of a test material, which is 80% active:

$$6 \text{ ppm} = \text{mg/kg} \times 100 / 80 = 7.5 \text{ mg/kg}$$

$$7.5 \text{ mg/kg} \times 20 \text{ kg} = 150 \text{ mg}$$

Therefore, mix 150 mg of test material in 20 kg of diet.

- Adjustment for high test material concentrations

When high concentrations of test material are used, displacement of diet must be considered and an

appropriate adjustment made. This is usually done when the amount of test material to be added is 5 g or more. The correction is made by subtraction of an equivalent weight of diet rounded to the nearest 10 g as shown in the following table:

Test Material (g)	Feed Displaced (g)
5.0–15.0	10
15.1–25.0	20
25.1–35.0	30
35.1–45.0	40
45.1–55.0	50

Example: To prepare 60 kg of a diet containing 30 g of test material (concentration of 500 ppm):

30 g of test material displaces 30 g of feed

60 kg of diet = 60,000 g of diet

60,000 g of diet – 30 g of test material = 59,970 g of feed

Therefore, mix 30 g of test material with 59,970 g of feed.

Adjustment of Concentration for Body Weight

Dietary concentrations are often adjusted to administer a specified amount of test material per unit of body weight each day, for example, mg/kg b.w./day. Each time the diet is prepared, adjustments are made based on body weight and food consumption data from the preceding interval(s), and the new concentration is calculated as follows:

- Calculation of dietary concentration

Dietary concentration (mg test material/kg diet) = desired dose (mg/kg b.w./day) \div predicted food consumption (g diet/kg b.w./day) \times 1000 (g/kg).

In our laboratory, predictions are made as follows:

- Predicted food consumption

The preceding interval's food consumption (expressed as grams of food consumed per animal per day) and the predicted body weight are used as follows: Predicted food consumption (g diet/kg b.w./day) = previous food consumption (g diet/day) \div predicted body weight (kg).

- Predicted body weight is calculated as follows: Predicted body weight (kg) = current weight (kg) \times 1.33 – previous weight (kg) \times 0.33.

Example: To calculate the dietary concentration needed to administer 100 mg of test material per kg b.w. per day during Week 3 to a group of rats which had a mean body weight of 200 g at the end of Week 1 and 249 g at the end of Week 2 and a mean food consumption value of 120 g of food per animal during a 6-day measurement period (20 g per animal per day) during Week 2.

- Calculate predicted body weight:

To predict the Week 3 weight of a group of rats which had mean weights of 200 g at the end of Week 1 and 249 g at the end of Week 2:

This week's (Week 2) weight \times 1.33: $249 \times 1.33 = 331 \text{ g}$

Last week's (Week 1) weight \times 0.33: $200 \times 0.33 = 66$ g
 Predicted Week 3 weight: $331 - 66 = 265$ g.

2. Calculate predicted food consumption:

$$\frac{\text{Previous week's f.c. : 20 g/day}}{\text{Predicted b.w. : 265 g}} \\ = 75.5 \text{ g diet/kg b.w./day}$$

3. Calculate dietary concentration:

$$\frac{100 \text{ mg t.m./kg b.w./day (dose)}}{75.5 \text{ g diet/kg b.w./day (predicted f.c.)} \times 1000} \\ = 1324.5 \text{ g t.m./kg diet}$$

Check: Based on prediction, a rat consumes 75.5 g/kg b.w./day of diet containing 1324.5 mg/kg of test material. $1324.5 \text{ mg test material/kg of diet} \times 0.0755 \text{ kg of diet} = 100 \text{ mg test material/kg b.w./day}$.

Test Material Intake (for Dietary Studies)

To calculate the actual test material intake (dose): Dose (mg of t.m./kg b.w./day) \times dietary concentration (mg t.m./g of diet) \times diet consumed (g diet/kg b.w./day).

Example: To determine the dose of test material received by a rat which consumed 120 g of diet/kg b.w./day of a diet containing 800 ppm of test material:

- $800 \text{ ppm} = 800 \text{ mg t.m./kg of diet} = 0.8 \text{ mg t.m./g of diet}$
- $0.8 \text{ mg t.m./g of diet} \times 120 \text{ g of diet/kg b.w./day} = 96 \text{ mg t.m./kg b.w./day}$

Approximate Conversion Factors (ppm to mg/kg/day)

When diets of a constant concentration are administered throughout a study, the actual test material intake per unit of body weight decreases as the animal grows older (and larger). Approximate conversion factors, assuming normal food consumption, to convert ppm in diet to mg test material/kg b.w./day are listed in Table 2.24.

TABLE 2.24

Conversion Factors (ppm to mg/kg)^a

Species	Age	Conversion Factor (Divide ppm by)
Mice	Young (1–12 weeks of study)	5
	Older (13–78 weeks of study)	6–7
Rats	Young (1–12 weeks of study)	10
	Older (13–104 weeks of study)	20
Dogs		40

^a Example: To estimate the approximate test material intake of rats receiving a 1000 ppm dietary concentration during a 4-week study: $1000 \text{ ppm} \div 10 = 100 \text{ mg/kg b.w./day}$.

CALCULATION OF TEST MATERIAL REQUIREMENTS

Amount of Material Needed for Capsule Administration or Dermal Administration of Neat Material

Sum of doses (mg/kg b.w./day) \times number of animals per group \times b.w. (kg)^a \times number of days.

Guidelines: Average body weight (kg).

Duration of Study	Rat	Mouse	Rabbit
	Sprague–Dawley	CD-1	NZW
<4 weeks	0.300	0.030	3
<12 weeks	0.400	0.035	4
>12 weeks	0.500	0.040	4
	Nonhuman primate cynomolgus	Dog	Minipig
<4 weeks	3	10	10
<12 weeks	3	10	15
>12 weeks	4	12	20

Notes: This is an approximate minimal amount. A safety factor of 20%–50% is usually added to allow for remixes, etc. Adjustment for active ingredient should be made if required.

Example: To calculate the amount of material needed for a 4-week study in dogs with doses of 10, 30, and 100 mg/kg b.w./day and a group size of 4 dogs/sex:

- Sum of doses $(10 + 30 + 100) = 140 \text{ mg/kg/day}$
- $140 \text{ mg/kg/day} \times 8 \text{ dogs} \times 10 \text{ kg} \times 30 \text{ days} = 336,000 \text{ mg (336 g)}$ with safety factor (additional 50%) request approximately 500 g.

Amount of Material Needed for Administration in Solution or Suspension

1. Calculate the amount of solution to be prepared at each interval (i.e., each mix): Dose (mL/kg b.w./day) \times b.w. \times number of animals \times number of days = mL/interval.

Example: To calculate the volume to be prepared to administer 10 mL/kg b.w./day to rats for a 6-month study in 20 rats per sex per group with dose solutions prepared weekly.

$$10 \text{ mL / kg b.w. / day} \times 0.500 \text{ kg per rats} \\ \times 40 \text{ rats} \times 8 \text{ days} = 1600 \text{ mL}$$

2. Calculate the amount of material to be used for each mix: Sum of concentrations (mg/mL) \times volume (mL) = amount needed (mg).

Example: To calculate test material needed when 1600 mL of dose solution per week per dose level are mixed, and dose concentrations are 20, 40, and 80 mg/mL:

- Sum of concentrations: $20 + 40 + 80 = 140 \text{ mg/mL}$
- $140 \text{ mg/mL} \times 1600 \text{ mL/week} = 224,000 \text{ mg (224 g)/week}$.

3. Calculate the amount of test material needed for the entire study: $\text{mg or g/interval} \times \text{number of intervals} = \text{mg or g needed}$.

Example: To calculate the total amount of material needed for the examples presented earlier: $224 \text{ g/week} \times 27 \text{ weeks}$ (one additional week added for pretest mix) = 5824 g, with safety factor (additional 20%), request approximately 7000 g.

Amount of Material Needed for Dietary Administration

1. Calculate the amount of dietary mixture to be prepared at each interval (i.e., each mix): Amount of feed per animal per interval \times number of feeders to be prepared. The amount of feed depends on the size of feeders. In our laboratory, the following are used: mice, 64 g/week; rats, 275 g/week; dogs, 3.5 kg/week (400 g/day).

In our laboratory, we prepare 5%–10% more feeders than needed for rodents (minimum of two extra feeders per group) and one extra feeder per dog per week.

Example: To calculate the amount of diet to be mixed each week for a group of 10 rats per sex:

$$275 \text{ g of feed/rat/week} \times 20 \text{ rats} = 5500 \text{ g} + 10\%$$

$$(\text{two additional feeders}) = 550 \text{ g}$$

$$\text{Total} = 6000 \text{ g}$$

A safety factor of 500–1000 additional grams may be added here.

2. Calculate the amount of test material to be used for each batch of diet to be mixed: Sum of concentrations (mg t.m./kg diet) \times batch size (kg) = mg of t.m. needed per batch.
 - a. For studies with the same concentration throughout the study: Sum of concentrations = sum of dose levels.
Example: To calculate the amount of material needed per week to administer dietary concentrations of 100, 200, and 400 ppm to groups of 10 rats per sex.
 - Sum of concentrations (100 + 200 + 400) = 700 ppm (mg t.m./kg diet)
 - 700 mg t.m./kg diet \times 6 kg diet = 4200 mg (4.2 g)
 - b. For studies with adjustment of concentration based on body weights and food consumption:

Sum of concentrations =

$$\frac{\text{Sum of dose levels (mg/kg/day)}}{\text{Predicted feed consumed (g/kg b.w./day)} \times 1000}$$

The following numbers, based on historical control data, are used in our laboratory for estimating the predicted feed consumed.

Duration of Study (Weeks)	Amount of Feed Consumed (g feed/kg b.w./day)	
	Rat	Mouse
4	100	250
12	80	215
26	70	190
>26	50	165

Example: To calculate the amount of material needed per week to administer doses of 50, 250, and 1250 mg/kg b.w./day to groups of 10 rats per sex for 4 weeks.

- Sum of concentrations (50, 250, and 1250) = 1550 mg/kg b.w./day
- $\frac{1550 \text{ mg/kg b.w./day}}{100 \text{ g/kg b.w./day} \times 1000} = 0.1155 \text{ mg/kg diet}$
- $0.1155 \text{ mg t.m./kg diet} \times 6 \text{ kg diet} = 693 \text{ mg}$

3. Calculate the amount of test material needed for the entire study: $\text{mg or g/interval} \times \text{number of intervals} = \text{mg or g needed}$.

Example (a): To calculate the total amount of test material needed for a 13-week study in which dietary concentrations of 100, 200, and 400 ppm are administered to groups of 10 rats per sex.

$$4.2 \text{ g/week} \times 14 \text{ weeks}$$

(one additional week added for pretest mix)

$$= 58.8 \text{ g,}$$

with safety factor (additional 20%–25%), request approximately 70–75 g.

Example (b): To calculate the total amount of test material needed for a 4-week study in which dietary doses of 50, 250, and 1250 mg/kg b.w./day are administered to groups of 10 rats/sex.

$$693 \text{ mg/week} \times 5 \text{ week}$$

(one additional week added for pretest mix)

$$= 3465 \text{ mg (3.465 g),}$$

with safety factor (additional 50%), request approximately 5 g.

Examples of Calculation Sheets Used in Our Laboratory

1. Test material calculations for a constant-concentration dietary study.

The study has four groups with 50 rats/sex/group. The dose levels (concentrations) are 0, 30, 100, and 300 ppm.

Test Material Calculation Sheet: Constant Concentration Diet

Duration: 105 weeks Sponsor code: _____
 Test material: _____ Study no.: _____
 Percent active: 100 AICF = 100% active: 1

Batch Size Calculation

Groups	No. of Feeders/Group ^a	kg (Feed)/Animal/ Week	Calculated Batch Size
I-IV	110	× 0.275	30.25 kg
Mixed Batch Size: 31.5 kg/group/week			

Test Material/Batch Calculations

Group	Dose Level		×	AICF	×	Mixed Batch Size (kg)	=	Test Material per Batch
	(ppm)	(mg/kg)						
II	30	30	×	1	×	31.5 ÷ 1000	=	0.945 g/week
III	100	100	×	1	×	31.5 ÷ 1000	=	3.15 g/week
IV	300	300	×	1	×	31.5 ÷ 1000	=	9.45 g/week
Total							=	13.545 g/week

Total Test Material Requirements

Total per Batch	×	No. of Mixes	×	Safety Factor	=	Test Material Required
13.545 g	×	106	×	1.2 ÷ 1000	=	1.7 kg

AICF, active ingredient correction factor.

^a Includes extra feeders.

2. Test material calculations for an adjusted-concentration dietary study.

The study has four groups with 50 rats/sex/group. The dose levels are 0, 30, 100, and 300 mg test material/kg/day.

Test Material Calculation Sheet: Adjusted Concentration Diet Calculations

Test Material: _____ Sponsor code: _____
 Percent active: 98% Study no.: _____
 AICF = 100% Study duration: 105 weeks
 active: 1.02

Batch Size Calculations

Group and Sex	No. of Feeders/ Sex/Group ^a	kg (Feed Presented) Animal/Week	Calculated Batch Size	Mixed Batch Size
I-IV	55	× 0.275	= 15.1 kg	16.5 kg

Amount of Test Material Required for Study Completion

$$\frac{\text{Sum of the dose level (mg/kg/day)}}{1000 \times \text{amount consumed/animal/day (g/kg/day)}} \times \text{Combined mixed batch size (male + female) in kilograms} \times \text{No. of weeks}$$

$$\text{AICF} \times \frac{\text{Safety factor}}{\text{factor}} = \text{Test material required}$$

$$\frac{430}{1000 \times 50} \times 33 \times 106 \times 1.02 \times 1.2 = 37 \text{ kg}$$

AICF, active ingredient correction factor.

^a Includes extra feeders.

dose volume is 5 mL/kg. The test material formulation is prepared on a weekly basis.

The study has four groups with 10 dogs/sex/group. The dose levels are 0, 30, 100, and 300 mg/kg/day, and the

Test Material Calculations: Oral Intubation Study						
Study duration:	13 weeks	Sponsor code:	_____			
Test material:	_____	Study No.:	_____			
Percent active:	100	AICF = 100% active:	1			
Batch size calculations						
Group and Sex	b.w. (kg)	Dose Volume (mL/kg)	No. of Animals per Goup	No. of Doses	Batch Size	Mixed Batch Size (mL)
II–IV	12	× 5	× 20	× 7	= 8400	= 10,080
Test Material/Batch Calculations						
Group	Dose Level (mg/kg day)	Dose Volume (mL/kg)	Conc. (mg/mL)	Batch (mL)	AICF	Test Material (g)
II	30	÷ 5	= 6	× 10,080	× 1 ÷ 1000	= 60.48
III	100	÷ 5	= 20	× 10,080	× 1 ÷ 1000	= 201.60
IV	300	÷ 5	= 60	× 10,080	× 1 ÷ 1000	= 604.80
				Total per batch	= 866.88	
Total Test Material Requirements						
Total per Batch (kg)	×	No. of Mixes	×	Safety Factor	=	Test Material Required (kg)
0.867	×	14	×	1.2	=	15
AICF, active ingredient correction factor.						

PROCEDURES FOR PREPARATION OF DOSE SOLUTIONS/SUSPENSIONS AND TEST DIETS

The following are samples of standard procedures used in our laboratory. Individual procedures are developed as needed, based on the properties of the test material. Preliminary analyses are performed to confirm the adequacy of preparation procedure.

Preparation of Dose Solutions/Suspensions

Materials: Test material
Vehicle

Equipment:
Balances
Beakers (or volumetric flasks)
Weighing spatulas
Glassine weigh paper of weigh boats
Sonicator
Stir plates and stir bars (if needed)
Calculation sheet

Procedure (Sample) for Suspension Preparation

1. Remove vehicle (methylcellulose) from refrigerator 1 h before mixing.
2. Using a weigh boat or weigh paper, weigh out the specified amount of test material (as per the current calculation sheet). Carefully transfer the test material into a 50 mL beaker.
3. Add approximately 2 mL of vehicle (methylcellulose) to the beaker, and begin stirring until a paste is formed. Continue adding vehicle (methylcellulose) to the beaker until reaching 3/4 of the total volume, rinsing off the spatula at this time.
4. Repeat steps 1 and 2 for each concentration to be prepared.
5. Place the beakers in a sonicator for approximately 15 min.
6. Remove the beakers from the sonicator and Q.S. each suspension with methylcellulose. Add a stir bar to the beakers, and allow the suspensions to stir on the stir plates for approximately 30 min.
7. Transfer to appropriately labeled storage containers.

Preparation of Test Diets

Materials: Test material
Certified rodent diet No. 5002 meal

Equipment:
Balances
Scales
Mixers: mortar/pestle, Hobart mixer, Twinshell mixer
Weigh boats or glassine weigh paper
Weighing spatulas
Appropriately labeled test material transfer containers
Appropriately labeled diet storage buckets
Calculation sheet

Procedure (Sample)

1. Weigh out the specified amount of test material using a weigh boat or weigh paper and transfer to an appropriate container.
2. Weigh out the specified amount of diet into a diet storage bucket.
3. Prepare an initial premix as follows: Place approximately 10 g of untreated feed from the diet storage bucket into a mortar. Pour the test material into the layer of feed in the mortar. Rinse the test material container with several grams of untreated feed (from the diet bucket), and add this rinse to the mortar. Pestle mixture until homogeneous.
4. Prepare an additional premix as follows: Place approximately 2 kg of untreated feed (from the diet bucket) into the bowl of the Hobart mixer. Pour the initial premix (from the mortar) into the Hobart mixer bowl. Rinse the mortar and pestle with several grams of untreated diet (from the bucket) to incorporate any residual test material, and add this rinse to the bowl of the Hobart mixer. Pour an additional 2.0 kg of diet (from the diet bucket) into the bowl of the Hobart mixer. Using the paddle blade, run the Hobart mixer on speed 1 for approximately 10 min.
5. Complete the mix as follows: Pour approximately half the diet remaining in the bucket into a Twinshell mixer. Add the premix from the Hobart mixer and rinse the Hobart bowl with several grams of untreated diet (from the diet bucket); add this rinse to the Twinshell. Pour the remaining diet into the Twinshell mixer and run the mixer for approximately 15 min. On completion, empty the mix into the appropriate dose group bucket (lined with a plastic bag). Attach the dose group identification tag to the bucket. Repeat for each concentration.

For both:

Handling Precautions (Specified as Necessary):

Respirator type:	Disposable dust/mist facemask
Gloves/hand protection:	Natural rubber or latex
Eye protection:	Safety glasses with sideshields
Foot protection:	Disposable shoe covers
Head protection:	Bouffant cap
Outer clothing:	White Tyvek suit or gray lab coat

Other procedures:

Sterile preparation in laminar flow hood.
Procedures for light-sensitive materials (yellow light, foil-covered sample containers).

ANALYSES OF DOSING SOLUTIONS/SUSPENSIONS AND DIETS

The following are the recommended procedures.

Method Validation

Analytical methods are developed and validated in the testing facility's laboratory. The designated vehicle (or diet) is mixed with the test material at concentrations over the range expected to be administered during the study. Mixtures are assayed according to the proposed method. The data obtained are evaluated for reproducibility of results. Means and standard deviations are computed for multiple extractions and injections of the low- and high-concentration mixtures. Modifications are made as necessary until acceptable results are obtained.

Homogeneity Analyses

These are required unless the mixture is a true solution, which by definition, is homogeneous. Before initiation of the study, batches of the low- and high-concentration mixtures are prepared. Three samples each from the top, middle, and bottom portion of each mix are taken for analysis. If the data demonstrate that the mean of the values for the three levels are within $\pm 10\%$ (solutions) or $\pm 15\%$ (diets) of each other and of the nominal (desired) concentration, the batch is considered homogeneous.

Stability Analyses

Stability of the test material in the mixture under storage conditions to be used for the study is determined for at least 2 weeks. Duplicate samples of the low- and high-concentration mixtures are assayed 4, 7, and 14 days after preparation (samples for homogeneity assays, evaluated on the day of preparation, are used to establish concentration at the time of preparation). If the data indicate that the test material is unstable at room temperature, frozen or refrigerated storage stability is evaluated.

Confirmation of Concentrations during Study

Solutions/suspensions or diets for all dose levels are assayed periodically (one sample per concentration is taken and two subsamples are analyzed). Suggested intervals are as follows:

1–4-week studies	First and last weeks
3-month studies	Weeks 1, 8, and 13
6- and 9-month studies	Weeks 1, 13, 26, and 39
24-month studies	Weeks 1 and 13, then quarterly

The concentration determined in the batch must be within $\pm 10\%$ (solutions) or $\pm 15\%$ (suspensions, diets) of each other before the mixtures are acceptable. If mixtures are not within the acceptable range, new mixtures are prepared and analyzed.

Summary

The number of analyses performed is as follows:

Homogeneity analyses		
9 samples per concentration \times 2 concentrations	= 18	
1 control sample (vehicle)	= 1	
Total	= 19	
Stability analyses		
2 samples per concentration \times 2 concentrations \times 3 intervals	= 12	
1 control sample \times 3 intervals	= 3	
Total	= 15	
Concentration	4-weeks study	24-months study
2 samples/concentration \times 3 concentrations \times 3–11 intervals	= 18	66
1 control sample \times 3–11 intervals	= 3	11
Total	= 21	77

EXPERIMENTAL EVALUATIONS

CLINICAL SIGNS OF TOXICITY (TABLES 2.25 THROUGH 2.27)

TABLE 2.25
Clinical Signs of Toxicity

Clinical Observation	Observed Signs	Organs, Tissues, or Systems Most Likely to Be Involved
I. Respiratory: blockage in the nostrils, changes in rate and depth of breathing, changes in color of body surfaces	A. Dyspnea: difficult or labored breathing; essentially gasping for air; respiration rate usually slow	CNS respiratory center, paralysis of costal muscles, cholinergic inhibition
	1. Abdominal breathing: breathing by diaphragm, greater deflection of abdomen upon inspiration	CNS respiratory center, pulmonary edema, secretion accumulation in airways (increase cholinergic)
	2. Gasping: deep labored inspiration, accompanied by a wheezing sound	CNS respiratory center, pulmonary cardiac insufficiency
	B. Apnea: a transient cessation of breathing following a forced respiration	Pulmonary–cardiac insufficiency, pulmonary edema
	C. Cyanosis: bluish appearance of tail, mouth, foot pads	Stimulation of respiratory center, pulmonary–cardiac insufficiency
D. Tachypnea: quick and usually shallow respiration		Pulmonary edema, hemorrhage
	E. Nostril discharges: red or colorless	

(continued)

TABLE 2.25 (continued)
Clinical Signs of Toxicity

Clinical Observation	Observed Signs	Organs, Tissues, or Systems Most Likely to Be Involved
II. Motor activities: changes in frequency and nature of movements	A. Decrease or increase in spontaneous motor activities, curiosity, preening, or locomotions	Somatomotor, CNS
	B. Somnolence: animal appears drowsy, but can be aroused by prodding and resumes normal activities	CNS sleep center
	C. Loss of righting reflex: loss of reflex to maintain normal upright posture when placed on the back	CNS, sensory, neuromuscular
	D. Anesthesia: loss of righting reflex and pain response (animal will not respond to tail and toe pinch)	CNS, sensory
	E. Catalepsy: animal tends to remain in any position in which it is placed	CNS, sensory, neuromuscular, autonomic
	F. Ataxia: inability to control and coordinate movement while animal is walking with no spasticity, epraxia, paresis, or rigidity	CNS, sensory, autonomic
	G. Unusual locomotion: spastic, toe walking, pedaling, hopping, and low body posture	CNS, sensory, neuromuscular
	H. Prostration: immobile and rests on belly	CNS, sensory, neuromuscular
	I. Tremors: involving trembling and quivering of the limbs or entire body	Neuromuscular, CNS
	J. Fasciculation: involving movements of muscles, seen on the back, shoulders, hind limbs, and digits of the paws	Neuromuscular, CNS, autonomic
III. Convulsion (seizure): marked involuntary contraction or seizures of contraction of voluntary muscle	A. Clonic convulsion: convulsive alternating contraction and relaxation of muscles	CNS, respiratory failure, neuromuscular, autonomic
	B. Tonic convulsion: persistent contraction of muscles, attended by rigid extension of hind limbs	
	C. Tonic-clonic convulsion: both types may appear consecutively	
	D. Asphyxial convulsion: usually of clonic type, but accompanied by gasping and cyanosis	
	E. Opisthotonos: tetanic spasm in which the back is arched and the head is pulled toward the dorsal position	
IV. Reflexes	A. Corneal (eyelid closure): touching of the cornea causes eyelids to close	Sensory, neuromuscular
	B. Pinnal: twitch of external ear elicited by light stroking of inside surface of ear	Sensory, neuromuscular, autonomic
	C. Righting	CNS, sensory, neuromuscular
	D. Myotact: ability of animal to retract its hind limb when limb is pulled down over the edge of a surface	Sensory, neuromuscular
	E. Light (pupillary): constriction of pupil in the presence of light	Sensory, neuromuscular, autonomic
	F. Startle reflex: response to external stimuli such as touch, noise	Sensory, neuromuscular
V. Ocular signs	A. Lacrimation: excessive tearing, clear or colored	Autonomic
	B. Miosis: constriction of pupil regardless of the presence or absence of light	Autonomic
	C. Mydriasis: dilation of pupils regardless of the presence or absence of light	Autonomic
	D. Exophthalmos: abnormal protrusion of eye from orbit	Autonomic
	E. Ptosis: dropping of upper eyelids, not reversed by prodding animal	Autonomic
	F. Chromodacryorrhea (red lacrimation)	Autonomic, hemorrhage, infection
	G. Relaxation of nictitating membrane	Autonomic
	H. Corneal opacity, iritis, conjunctivitis	Irritation of the eye
VI. Cardiovascular signs	A. Bradycardia: decreased heart rate	Autonomic, pulmonary–cardiac insufficiency
	B. Tachycardia: increased heart rate	Autonomic, pulmonary–cardiac insufficiency
	C. Vasodilation: redness of skin, tail, tongue, ear, foot pad, conjunctivae, and warm body	Autonomic, CNS, increased cardiac output, hot environment

TABLE 2.25 (continued)
Clinical Signs of Toxicity

Clinical Observation	Observed Signs	Organs, Tissues, or Systems Most Likely to Be Involved
	D. Vasoconstriction: blanching or whitening of skin, cold body	Autonomic, CNS, cold environment, cardiac output decrease
	E. Arrhythmia: abnormal cardiac rhythm	CNS, autonomic, cardiopulmonary insufficiency, myocardial infarction
VII. Salivation	A. Excessive secretion of saliva: hair around mouth becomes wet	Autonomic
VIII. Piloerection	A. Contraction of erectile tissue of hair follicles resulting in rough hair	Autonomic
IX. Analgesia	A. Decrease in reaction to induced pain (e.g., hot plate)	Sensory, CNS
X. Muscle tone	A. Hypotonia: generalized decrease in muscle tone	Autonomic
	B. Hypertonia: generalized increase in muscle tension	Autonomic
XI. Gastrointestinal signs: droppings (feces)	A. Solid, dried, and scant	Autonomic, constipation, GI motility
	B. Loss of fluid, watery stool	Autonomic, diarrhea, GI motility
Emesis	A. Vomiting and retching	Sensory, CNS, autonomic (in rat, emesis is absent)
Diuresis	A. Red urine (hematuria)	Damage in kidney
	B. Involuntary urination	Autonomic, sensory
XII. Skin	A. Edema: swelling of tissue filled with fluid	Irritation, renal failure, tissue damage, long term immobility
	B. Erythema: redness of skin	Irritation, inflammation, sensitization

Source: Chan, P.K. and Hayes, A.W., Principles and methods for acute toxicity and eye irritancy, in *Principles and Methods of Toxicology*, 2nd edn., Raven Press, New York, 1989. With permission.

TABLE 2.26
Autonomic Signs

Sympathomimetic	Piloerection
	Partial mydriasis
Sympathetic block	Ptosis
	Diagnostic if associated with sedation
Parasympathomimetic	Salivation (examined by holding blotting paper)
	Miosis
	Diarrhea
	Chromodacryorrhea in rats
Parasympathomimetic block	Mydriasis (maximal)
	Excessive dryness of mouth (detect with blotting paper)

Source: Chan, P.K. and Hayes, A.W., Principles and methods for acute toxicity and eye irritancy, in *Principles and Methods of Toxicology*, 2nd edn., Raven Press, New York, 1989. With permission.

TABLE 2.27
Toxic Signs of Acetylcholinesterase Inhibition

Muscarinic Effects ^a	Nicotinic Effects ^b	CNS Effects ^c
Bronchoconstriction	Muscular twitching	Giddiness
Increased bronchorecretion	Fasciculation	Anxiety
Nausea and vomiting (absent in rats)	Cramping	Insomnia
	Muscular weakness	Nightmares
Diarrhea		Headache
Bradycardia		Apathy
Hypotension		Depression
Miosis		Drowsiness
Urinary incontinence		Confusion
		Ataxia
		Coma
		Depressed reflex
		Seizure
		Respiratory depression

Source: Chan, P.K. and Hayes, A.W., Principles and methods for acute toxicity and eye irritancy, in *Principles and Methods of Toxicology*, 2nd edn., Raven Press, New York, 1989. With permission.

^a Blocked by atropine.

^b Not blocked by atropine.

^c Atropine might block early signs.

HISTORICAL CONTROL DATA—RODENT BODY WEIGHTS

Tables 2.28 and 2.29 show representative mean values from several chronic studies conducted in our laboratory. (Age at week-1 is approximately 5 weeks.)

TABLE 2.28
Body Weights (g) of Control CD1
Mice in Chronic Toxicity Studies
Conducted between 2006 and 2011

Week	Males	Females
-1	30.0	23.0
0	31.5	24.0
1	31.5	24.4
2	32.2	24.8
3	32.8	25.4
4	32.8	26.1
5	34.2	26.4
6	34.9	26.8
7	35.7	27.3
8	36.1	27.6
9	36.4	27.9
10	37.0	28.1
11	37.3	28.4
12	37.5	28.7
13-16	39.0	29.2
17-21	39.5	29.7
22-25	39.6	29.9
26-29	40.3	30.0
30-33	40.1	30.2
34-37	40.9	31.0
38-41	41.5	31.2
42-45	41.7	31.7
46-49	42.0	31.9
50-53	41.9	32.3
54-57	42.2	32.6
58-61	42.6	33.0
62-65	42.4	33.1
66-69	42.4	33.2
70-73	42.3	33.5
74-77	42.0	34.0
78-81	42.2	34.3
82-85	42.1	34.4
86-89	41.8	34.5
90-93	41.6	34.5
94-97	41.4	34.7
98-101	41.8	34.5

TABLE 2.29
Body Weights (g) of Control Sprague-
Dawley Rats in Chronic Toxicity Studies
Conducted between 2006 and 2011

Week	Males	Females
-1	141	135
0	214	173
1	259	193
2	309	216
3	353	233
4	392	247
5	424	257
6	451	268
7	474	274
8	494	283
9	510	288
10	528	293
11	542	298
12	556	304
13	569	308
14	596	316
15	630	334
20-25	663	350
26-29	690	374
30-33	714	382
34-37	741	399
38-41	759	415
42-45	787	432
46-49	802	447
50-53	813	462
54-57	821	475
58-61	833	488
62-65	841	500
66-69	844	510
70-73	851	520
74-77	857	524
78-81	858	534
86-89	854	540
90-93	845	548
94-97	845	549
98-101	831	553
101-102	810	536

EFFECT OF DECREASED BODY WEIGHTS ON ORGAN WEIGHTS OF RATS (TABLE 2.30)

TABLE 2.30
Effect of Decreased Body Weights
on Relative Organ Weights^a of Rats^b

Decrease	No Change	Increase
Liver (?)	Heart	Adrenal glands (?)
	Kidneys	Brain
	Prostate	Epididymides
	Spleen	Pituitary
	Ovaries	Testes
		Thyroid (?)
		Uterus

Note: ?, Differences slight or inconsistent.

^a Relative weights: organ/body weight ratios.

^b For absolute weights, all except thyroids decrease. Summary of results reported in Schwartz, E.R. et al.,⁷ and Scharer, K.⁸

RODENT SURVIVAL RATES (TABLES 2.31 AND 2.32)

TABLE 2.31
Monthly Survival Rates of Control CD1 Mice in
Chronic Toxicity Studies Conducted between 2006
and 2011

Month of Study	Males		Females	
	Mean % Survival		Mean % Survival	
	Oral Gavage	Inhalation	Oral Gavage	Inhalation
1	100	100	100	100
2	98	100	100	100
3	98	100	100	100
4	98	93	99	96
5	97	87	98	90
6	97	82	97	82
7	97	77	97	78
8	96	71	97	71
9	95	68	95	66
10	94	66	95	66
11	93	65	93	64
12	91	63	92	63
13	89	62	90	61
14	86	60	88	57
15	83	56	85	56
16	80	53	82	54
17	75	49	80	50
18	71	44	76	45
18	65	42	71	41
20	59	36	65	40
21	55	33	59	36
22	51	28	53	35
23	44	24	44	31
24	38	23	40	27

TABLE 2.32
Monthly Survival Rates of Control Sprague–
Dawley Rats in Chronic Toxicity Studies
Conducted between 2006 and 2011

Month of Study	Males	Females
	Mean % Survival	Mean % Survival
1	100	100
2	100	100
3	100	100
4	100	100
5	99	100
6	98	99
7	97	99
8	97	99
9	96	99
10	95	98
11	93	98
12	92	97
13	89	95
14	87	91
15	83	88
16	77	85
17	70	80
18	65	74
19	56	67
20	52	61
21	46	56
22	45	46
23	42	40
24	44	37

REFERENCES

1. Smith, D., Combes, R., Depelchin, O., Jacobsen, S., Hack, R., Luft, J., Lammens, L., Von Landenberg, F., Phillips, B., Pfister, R., Rabemampianina, Y., Sparrow, S., Stark, C., Stephan-Gueldner, M., Optimising the design of preliminary toxicity studies for pharmaceutical safety testing in the dog. *Reg. Tox. Pharm.* 41 (2005) 95–101.
2. Gad, S., Cassidy, C., Aubert, N., Spainhour, B., Robbe, H., Nonclinical vehicle use in studies by multiple routes in multiple species. *IJT* 25 (2006) 499–521.
3. *SYNAPSE*, American Society of Laboratory Animal Practitioners, Vol. 24, 1991.
4. *Laboratory Manual for Basic Bi methodology of Laboratory Animals*, MTM Associates, Inc.
5. Freireich, E.J. et al., Quantitative comparison of toxicity of anti-cancer agents in mouse, rat, dog, monkey and man. *Cancer Chemother. Rep.* 50 (1966) 219.
6. Chan, P.K., Hayes, A.W., Principles and methods for acute toxicity and eye irritancy. In *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., ed., Raven Press, New York, 1989.
7. Schwartz, E., Tomaben, J.A., Boxill, G.C., The effects of food restriction on hematology, clinical chemistry and pathology in the albino rat. *Toxicol. Appl. Pharmacol.* 25 (1973) 515.
8. Scharer, K., The effect of underfeeding on organ weights of rats. How to interpret organ weight changes in cases of marked growth retardation in toxicity tests. *Toxicology* 7 (1977) 45.

3 Dermal Toxicology

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INTRODUCTION

This chapter presents information on both classical and current *in vivo* methods for evaluating dermal irritation and sensitization potential of chemicals, drugs, and consumer products. Some methods that originated in the 1940s and 1950s are still used today. However, over the last several decades, animal welfare concerns have resulted in the refinement of classical methods and development of newer *in vivo* methods that have gradually replaced the older methods.

Various committees, most notably the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) under the National Toxicology Program (NTP) Interagency Center for the Evaluation of

Alternative Toxicological Methods (NICEATM) as well as other committees, examine the validity and appropriate application of alternative toxicology methods. These committees coordinate technical review and evaluation of new and revised test methods. They work on harmonization of methodologies and classifications internationally. They report their findings to policy-making agencies for advancing techniques to promote animal welfare with emphasis on the 3 R's of reduction, refinement, and replacement of animal testing (where feasible) while maintaining public health. Several new methodologies have been generally accepted and implemented as a result of the work of these various committees including the Mouse Local Lymph Node Assay as an alternative, when appropriate, to other

sensitization assays and *in vitro* screening assays for corrosivity, phototoxicity testing, and sensitization. The *in vitro* assays are covered in a separate chapter. In addition, various agencies and their committees, such as the European Union Joint Meeting of the Chemicals Committee and the Working Party on Chemicals as well as the United Nations,

have examined country differences and are working toward more unified classification systems such as the Globally Harmonized System.

COMPARISON OF MAJOR STUDY DESIGNS (TABLES 3.1 AND 3.2)

TABLE 3.1
Comparison of Dermal Irritation Study Designs

Category	EPA-OPPTS (870.2500)	JMAFF
Number of animals	Generally three healthy adults, recommend initiating with one animal followed by two more pending results.	At least three young adults
Species	Albino rabbit recommended	White rabbit; may be initiated with a single animal if needed
Preliminary screens	May not test if: $\text{pH} \leq 2$, ≥ 11.5 , dermal $\text{LD}_{50} < 200$ mg/kg, dermal limit test at 2000 mg/kg did not produce irritation, a validated and accepted <i>in vitro</i> test demonstrates corrosive properties, or corrosive potential is predicted from structure–activity relationships.	May not test if: $\text{pH} \leq 2$, ≥ 11.5
Control group	None required	None required
Preparation of skin	Clip or shave ≈ 24 h before test Use only healthy, intact skin	Clip ≈ 24 h before test Use only healthy, intact skin
Application site	Dorsal area of trunk	Dorsal area of trunk
Application area	≈ 6 cm ²	≈ 6 cm ²
Patch types	Gauze patch held loosely in contact with skin using nonirritating tape	Gauze patch held in contact with skin using nonirritating tape
Occlusion	Semiocclusive dressing	Semiocclusive dressing preferred; occlusive may be appropriate
Preparation of test substance	Solids: pulverized if necessary, moistened sufficiently with water or suitable vehicle to ensure good skin contact Liquids: generally used undiluted	Solids: crushed if necessary, moistened thoroughly with water or suitable vehicle to ensure good skin contact Liquids: applied undiluted
Dose level	Solids: 0.5 g Liquids: 0.5 mL	Solids: 0.5 g Liquids: 0.5 mL
Exposure interval	4 h (3 min, 1 h if corrosion is anticipated)	4 h (3 min, 1 h, 4 h to the first animal if severe potential irritation/corrosion is anticipated)
No. of applications	One	One
Test substance residue removal	Water or another appropriate solvent	Water or another appropriate solvent
Observation intervals	30–60 min, 24, 48, and 72 h after patch removal	30 or 60 min, 24, 48, and 72 h after patch removal
Minimum observation period	72 h	72 h
Maximum observation period	14 days	14 days
Category	European-OECD	European-EEC (Harmonized with OECD)
Number of animals	Generally three healthy adults, recommended to initiate with one animal followed by one or two more pending results	Generally three healthy adults, recommended to initiate with one animal followed by one or two more pending results
Species	Albino rabbit recommended	
Preliminary screens	Do not test if: $\text{pH} \leq 2$, ≥ 11.5 , material is highly toxic by dermal route, dermal limit test at 2000 mg/kg did not produce irritation, <i>in vitro</i> test indicates corrosive properties	Do not test if: $\text{pH} \leq 2$, ≥ 11.5 , material is highly toxic by dermal route, dermal limit test at 2000 mg/kg did not produce irritation, <i>in vitro</i> test indicates corrosive properties
Control group	None required	None required
Preparation of skin	Closely clip ≈ 24 h before test; use only healthy, intact skin	Clip or shave ≈ 24 h before test; use only healthy, intact skin
Application site	Dorsal area of trunk	Dorsal area of trunk
Application area	≈ 6 cm ²	≈ 6 cm ²

TABLE 3.1 (continued)
Comparison of Dermal Irritation Study Designs

Category	EPA-OPPTS (870.2500)	JMAFF
Patch types	Gauze patch held loosely in contact with skin using nonirritating tape	Gauze patch held loosely in contact with skin using nonirritating tape
Occlusion	Semiocclusive	Semiocclusive
Preparation of test substance	Solids: Pulverized if necessary, moistened with less amount of water or suitable vehicle to ensure good skin contact Liquids: Applied undiluted	Solids: Pulverized if necessary, moistened sufficiently with water or suitable vehicle to ensure good skin contact Liquids: Applied undiluted
Dose level	Solids: 0.5 g Liquids: 0.5 mL	Solids: 0.5 g Liquids: 0.5 mL
Exposure interval	4 h; may be reduced to 1 h or 3 min	4 h; may be reduced to 1 h or 3 min
No. of applications	One	One
Test substance residue removal	Water or another appropriate solvent	Water or another appropriate solvent
Observation intervals	60 min, 24, 48, and 72 h after patch removal (also at patch removal for first animal)	60 min, 24, 48, and 72 h after patch removal (also at patch removal for first animal)
Minimum observation period	72 h	72 h
Maximum observation period	14 days	14 days

Sources: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2500, Acute Dermal Irritation, August, 1998; Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Irritation Studies (2-1-4), November 13–14, 2000; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 404: Acute Dermal Irritation/Corrosion, 1, 2002; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.4. Acute Toxicity (Skin Irritation), 2008.

TABLE 3.2
Comparison of Sensitization Study Designs

Category	EPA OPPTS 870.2600	JMAFF
Acceptable test methods	Buehler Test (BT), ^a Guinea Pig Maximization Test (GPMT), ^a LLNA ^a	
Species	Guinea pig (BT or GPMT) Mouse (LLNA)	
Number and sex	BT: 20 per treated group; 10 per control group GPMT: 10 minimum per treated group; 5 minimum per control group but may need to add more pending results LLNA: minimum of 5 animals per dose group; individually housed	
Control animals	Periodic (every 6 months) use of a positive control substance with an acceptable level of reliability for the test system selected is recommended; irritation controls should be used [Guinea pig (BT or GPMT)] Concurrent recommended or minimum of once per month use of a positive control substance (e.g., HCA and mercaptobenzothiazole; benzocaine is not recommended) with an acceptable level of reliability for the test system selected is recommended; irritation controls should be used [Mouse (LLNA)]	
Dose level	Dependent on the method used	
Preparation of skin	Clipping, shaving, or depilation depending on the method used [Guinea pig (BT or GPMT)] None [Mouse (LLNA)]	
Observation of animals	Skin reactions are to be graded and recorded after the challenge exposure at the time specified by the methodology selected (usually 24, 48, and 72 h) (Guinea pig [BT or GPMT]) Overt local reactions and systemic toxicity and ear measurements if collected (Mouse [LLNA])	
Body weights	Initial and terminal body weights required	
Category	Japanese-MHW	JMAFF
Acceptable test methods	Adjuvant and patch test; BT ^b ; Draize test; FCA test; maximization test ^c ; open optimization test; split adjuvant test	BT, ^a GPMT ^b ; other methods may be used provided that they are well validated and scientific justification is given

(continued)

TABLE 3.2 (continued)
Comparison of Sensitization Study Designs

Category		EPA OPPTS 870.2600
Species	Guinea pig	Guinea pig
Number and sex	Dependent on the method used	Dependent on the method used
Control animals	Positive controls are required; preferred substances include <i>p</i> -phenylenediamine, 1-chloro-2,4-dinitrobenzene, neomycin sulfate, and nickel sulfate	Positive controls are required to evaluate the responsivity of the test system
Dose level	Dependent on the method used	Dependent on the method used
Preparation of skin	Dependent on the method used	Dependent on the method used
Observation of animals	Skin reactions are to be noted at 24, 48, or 72 h after challenge exposure	Skin reactions are to be graded and recorded after the challenge exposure at the time specified by the methodology selected (usually 24, 48, and 72 h)
Body weights	Initial and terminal body weights required	Initial and terminal body weights required
Category	European-OECD	European-EEC
Acceptable test methods	BT; ^a GPMT; ^a LLNA ^a ; other methods may be used provided that they are well validated and scientific justification is given	BT; ^a GPMT ^a ; other methods may be used provided that they are well validated and scientific justification is given
Species	Guinea pig (BT or GPMT) Mouse (LLNA)	Guinea pig (BT or GPMT) Mouse (LLNA)
Number and sex	BT: 20 per treated group; 10 per control group GPMT: 10 minimum per treated group; 5 minimum per control group but may need to add more pending results LLNA: minimum of 4 animals per dose group; group-housed	BT: 20 per treated group; 10 per control group GPMT: 10 minimum per treated group; 5 minimum per control group but may need to add more pending results LLNA: minimum of 4 or 5 animals per dose group; group-housed
Control animals	Mild-to-moderate positive controls are required every 6 months; preferred substances are HCA, mercaptobenzothiazol, and benzocaine; others are accepted with justification (Guinea pig [BT or GPMT]) Concurrent recommended or minimum of once per month use of a positive control (e.g., OECD 429: 25% HCA in acetone:olive oil as a 4:1 v/v or 5% mercaptobenzothazole in N,N-dimethylformamide; OECD 442A/442B: 25% HCA or 25% eugenol in acetone:olive oil as 4:1 v/v) substance with an acceptable level of reliability for the test system selected is recommended; irritation controls should be used (Mouse [LLNA])	Mild-to-moderate positive controls are required every 6 months; preferred substances are HCA, benzothiazole-2-thiol (mercaptobenzothiazole), benzocaine, or others that are known sensitizing substances from the literature (Guinea pig [BT or GPMT]) Concurrent recommended or no greater than once every 6 months use of a positive control (e.g., HCA or mercaptobenzothiazole) substance with an acceptable level of reliability for the test system selected is recommended; irritation controls should be used (Mouse [LLNA])
Dose level	Dependent on the method used	Dependent on the method used
Preparation of skin	Clipping, shaving, or depilation depending on the method used (Guinea pig [BT or GPMT]) Auricular pretreatment in OECD442A (Mouse [LLNA])	Clipping, shaving, or depilation dependent on the method used None (Mouse [LLNA])
Observation of animals	24 and 48 h after patch removal at challenge(s) (Guinea pig [BT or GPMT]) Auricular skin reaction and ear thickness measurements (Mouse [LLNA])	All skin reactions from induction and challenge procedures should be recorded and reported
Body weights	Initial and terminal body weights required	Initial and terminal body weights required

Sources: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2600: Skin Sensitization Study, 2003; Japan New Drugs Division Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, 1990 Guidelines for Toxicity Studies of Drugs Manual, 1991, Chapter 7; Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Sensitization Studies (2-1-6), November 16–18, 2000; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 406: Skin Sensitization, 1992; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.6: Skin Sensitization, 2008; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 429: Skin Sensitization: Local Lymph Node Assay, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442A: Skin Sensitization: Local Lymph Node Assay: DA, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, 2010; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.42: Skin Sensitization: Local Lymph Node Assay, 2008; U.S. Environmental Protection Agency, Office of Pesticide Programs: Expansion of the Traditional Local Lymph Node Assay for the Assessment of Dermal Sensitization Potential of End Use Pesticide Products and Adoption of a “Reduced” Protocol for the Traditional LLNA (Limit Dose), January 27, 2011.

^a These test methods are the most widely used.

COMPARISON OF REGULATORY GUIDELINES (TABLES 3.3 THROUGH 3.7)

TABLE 3.3
Quick Reference Chart for Common US Test Guidelines

Regulatory Group Dermal	Reference	Specific Section for	
		Dermal Irritation	Dermal Sensitization
US Environmental Protection Agency (EPA)	Health Effects Test Guidelines	OPPTS 870.2500 (August 1998)	OPPTS 870.2600 (March 2003)
US Consumer Product Safety Commission (CPSC)	Subchapter C—Federal Hazardous Substances Act (FHSA) Regulations, 16 CFR Part 1500, September 2012	Section 1500.41	Section 1500.3
US Department of Transportation (DOT)	49 CFR, Part 173, October 2004 (DOT)	Sections 173.136, 173.137	Not specified
US Food and Drug Administration (FDA)	Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food, Redbook II, 1993 (FDA)	Not specified	Not specified
US Pharmacopeia (USP)	The US Pharmacopeia, USP24 and The National Formulary, NF 19, January 1, 2000 (USP)	Chapter 88	Not specified

Sources: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2500, Acute Dermal Irritation, August, 1998; U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2600: Skin Sensitization Study, 2003; U.S. Environmental Protection Agency, Office of Pesticide Programs: Expansion of the Traditional Local Lymph Node Assay for the Assessment of Dermal Sensitization Potential of End Use Pesticide Products and Adoption of a “Reduced” Protocol for the Traditional LLNA (Limit Dose), January 27, 2011; U.S. Consumer Products Safety Commission, 16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.41: Method of Testing Primary Irritant Substances, September 2012; U.S. Consumer Products Safety Commission, 16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.3: Definitions, 382, September 2012; U.S. Research and Special Programs Administration, Department of Transportation, 49 CFR, Part 173. 136 and 137, September 2012; U.S. Pharmacopeia, National Formulary, USP 24 NF 19 Biological Reactivity Test, INVIVO, 1832, 2000, Chapter 88.

TABLE 3.4
Quick Reference Chart for Common Foreign Test Guidelines

Regulatory Group	Reference	Specific Section for	
		Dermal Irritation	Dermal Sensitization
Government of Canada, Environment Canada, and Health and Welfare Canada (CEPA)	Canadian Environmental Protection Act, Guidelines for the Notification and Testing of New Substances: Chemicals and Polymers, March 1993	Section 5.1	Section 5.1
European Organization for Economic Cooperation and Development (OECD)	Guidelines for Testing of Chemicals, Section 4, Health Effects	Subsection 404	Subsection 406 (July 1992) and 429, 442A, 442B (July 2010)
European Economic Community (EEC)	Part B: Methods for the Determination of Toxicity, May 2008	No. L 141/142, B.4	No. L 141/141, B.6

(continued)

TABLE 3.4 (continued)
Quick Reference Chart for Common Foreign Test Guidelines

Regulatory Group	Reference	Specific Section for	
		Dermal Irritation	Dermal Sensitization
Japanese Ministry of Health and Welfare (MHW)	1990 Guidelines for Toxicity Studies of Drugs Manual, September 1989	Not specified	Chapter 7, pp. 75–80
Global Harmonized System (United Nations)	Part 3, Health Hazards 2007	Chapter 3.1, pp. 121–132	Chapter 3.4, pp. 147–155

Sources: Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Irritation Studies (2-1-4), November 13–14, 2000; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 404: Acute Dermal Irritation/Corrosion, 1, 2002; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.4. Acute Toxicity (Skin Irritation), 2008; Japan New Drugs Division Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, 1990 Guidelines for Toxicity Studies of Drugs Manual, Chapter 7, 1991; Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Sensitization Studies (2-1-6), November 16–18, 2000; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 406: Skin Sensitization, 1992; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.6: Skin Sensitization, 2008; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 429: Skin Sensitization: Local Lymph Node Assay, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442A: Skin Sensitization: Local Lymph Node Assay: DA, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, 2010; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.42: Skin Sensitization: Local Lymph Node Assay, 2008; A Guide to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Part 3 Health Hazards, United Nations, 2007; Canadian Environmental Protection Act, Guidelines for the Notification and Testing of New Substances: Chemicals and Polymers, Section 5.1: Test Procedures and Practices, 50, 1993

TABLE 3.5
Quick Reference Chart for Miscellaneous Test Guidelines

Regulatory Group	Reference	Study Type
International Maritime Organization (IMO)	International Maritime Dangerous Goods Code	Dermal corrosion
Occupational Safety and Health Administration (OSHA)	OSHA's Hazard Communication Standard, 29 CFR 1900.1200, Appendix A, 2012	Dermal irritation and sensitization
American Society for Testing and Materials (ASTM)	Annual Book of ASTM Standards, F719 (13.01), E993 (11.04), F720 (13.01)	Dermal irritation and sensitization
The Cosmetic, Toiletry and Fragrance Association, Inc. (CTFA)	CTFA Safety Testing Guidelines, Sections II and IV	Dermal irritation and sensitization

Sources: International Maritime Dangerous Goods Code, Class 8 Corrosives, International Maritime Organization, London, U.K., 1994; Occupational Safety and Health Administration, Labor, 29 CFR Chapter XVII, Part 1910, Appendix A to Section 1900.1200: Health Hazard Definitions (Mandatory), 2012; American Society for Testing and Materials, 1991 Annual Book of ASTM Standards, F719–81 (1986), 13.01: Practice for Testing Biomaterials in Rabbits for Primary Skin Irritation, 976, 1991; American Society for Testing and Materials, 1991 Annual Book of ASTM Standards, F720–81 (1986), 13.01, Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test, 976, 1991; American Society for Testing and Materials, 1991 Annual Book of ASTM Standards, E993–88, 11.01, Test Method for Evaluation of Delayed Contact Hypersensitivity, 964, 1991; The Cosmetic, Toiletry and Fragrance Association, Inc., CTFA Safety Testing Guidelines, Section II: Guidelines for Evaluating Primary Skin Irritation Potential, 2, 1991; The Cosmetic, Toiletry and Fragrance Association, Inc., CTFA Safety Testing Guidelines, Section IV, Guidelines for Evaluating Contact Sensitization Potential, 7, 1991.

TABLE 3.6
Comparison of Excerpts from Selected Dermal Irritation Test Guidelines

EPA OPPTS 870.2500

- (a) *Scope* — (1) *Applicability*: This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 USC 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 USC 2601).
- (2) *Background*: The sources of materials used in developing this harmonized OPPTS test guideline are 40 CFR 798.4470 Primary Dermal Irritation; OPP 81-5 Primary Dermal irritation (Pesticide Assessment Guidelines, Subdivision F — Hazard Evaluation; Human and Domestic Animals); EPA report 540/09-82-025, 1982; and OECD 404 Acute Dermal Irritation/Corrosion.
- (b) *Purpose*: Determination of the irritant and/or corrosive effects on skin of mammals is useful in the assessment and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the substance.
- (c) *Definitions*: The definitions in section 3 of TSCA and in 40 CFR Part 792 — Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.
- “Dermal corrosion” is the production of irreversible tissue damage in the skin following the application of a test substance.
- “Dermal irritation” is the production of reversible inflammatory changes in the skin following the application of a test substance.
- “Pharmacological effect” means any chemically induced physiological changes in the test animal.
- “Target organ” means any organ of a test animal showing evidence of an effect of chemical treatment.
- (d) *Principle of the test methods*: (1) The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control [except when severe irritation/corrosion is suspected and the stepwise procedure is used (see paragraph (f)(1)(iii))]. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days.
- (2) When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.
- (e) *Initial considerations*: (1) Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less, or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties.
- (2) It is unnecessary to test materials which have been shown to be highly toxic (LD50 less than 200 mg/kg) by the dermal route or have been shown not to produce irritation of the skin at the limit test dose level of 2000 mg/kg body weight.
- (3) It may not be necessary to test in vivo materials for which corrosive properties are predicted on the basis of results from well validated and accepted in vitro tests. If an in vitro test is performed before the in vivo test, a description or reference to the test, including details of the procedure, must be given together with results obtained with the test and reference substances.
- (4) It may not be necessary to test materials for which corrosive potential is predicted from structure–activity relationships.
- (f) *Test procedures* — (1) Animal selection — (i) *Species and strain*. The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.
- (ii) *Number of animals*. At least three healthy adult animals (either sex) should be used unless justification/reasoning for using fewer animals is provided. It is recommended that a stepwise procedure be used to expose one animal, followed by additional animals to clarify equivocal responses.
- (iii) *Stepwise exposure of animals*. A single rabbit may be used if it is suspected that the test material might produce severe irritation/corrosion. Three test patches are applied concurrently or sequentially to the animal. The first patch is removed after 3 min. If no serious skin reaction is observed, the second patch is removed after 1 h. If observations indicate that exposure can be continued humanely, the third patch is removed after 4 h and the responses graded. If a corrosive effect is observed after an exposure of up to 4 h, then further animal testing is not required. If no corrosive effect is observed in one animal after a 4 h exposure, the test is completed using two additional animals, each with one patch only, for an exposure period of 4 h. If it is expected that the test substance will not produce severe irritancy or corrosion, the test may be started using three animals, each receiving one patch for an exposure period of 4 h.

(continued)

TABLE 3.6 (continued)

Comparison of Excerpts from Selected Dermal Irritation Test Guidelines

EPA OPPTS 870.2500

- (2) *Control animals*: Separate animals are not recommended for an untreated control group. Adjacent areas of untreated skin of each animal may serve as a control for the test.
- (3) *Dose level*: A dose of 0.5 mL of liquid or 500 mg of solid or semisolid is applied to the test site.
- (4) *Preparation of test area*: Approximately 24 h before the test, fur should be removed from the test area by clipping or shaving from the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.
- (5) *Application of the test substance*: (i) The recommended exposure duration is normally 4 h unless corrosion is observed [see paragraph (f)(1)(ii)]. Longer exposure may be indicated under certain conditions (e.g., expected pattern of human use and exposure). At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.
- (ii) When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. If a vehicle is used, it should not alter the absorption, distribution, metabolism, retention, or the chemical properties of the test substance, nor should it enhance, reduce, or alter its toxic characteristics. Although water or saline is the preferred agent to be used for moistening dry test materials, other agents may be used provided the use is justified. Acceptable alternatives include: gum arabic, ethanol and water, carboxymethyl cellulose, polyethylene glycol, glycerol, vegetable oil, and mineral oil.
- (iii) The test substance should be applied to a small area (approximately 6 cm²) of skin and covered with a gauze patch, which is held in place with a nonirritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semioclusive dressing for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.
6. *Observation period*: The duration of the observation period need not be rigidly fixed. It should be sufficient to fully evaluate the reversibility or irreversibility of the effects observed. It need not exceed 14 days after application.
7. *Clinical examination and scoring*: (i) After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30–60 min, and at 24, 48, and 72 h after patch removal.
- (ii) Dermal irritation should be scored and recorded according to the grades provided in the guidelines. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any lesions and other toxic effects should be fully described.
- (g) *Data and reporting* — (1) *Data summary*. Data should be summarized in tabular form, showing for each individual animal the irritation scores for erythema and edema at 30–60 min, and 24, 48, 72 h after patch removal, any other dermal lesions, a description of the degree and nature of the irritation, corrosion and reversibility, and any other toxic effects observed.

Japanese-MAFF

1. *Objective*

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used by obtaining scientific information regarding skin irritant properties and corrosiveness of the test substance.

2. *Test substance*

The preparation. However, this test should not be undertaken with strongly acidic or alkaline materials (generally those up to pH 2 and from pH 11.5) as these may be expected to be corrosive.

3. *Test animal species and age*

Use three or more young adult albino rabbits. Points to keep in mind: Conduct the test with only one animal if test substance has severe skin irritant properties.

4. *Administration method*

1. Clip the hair on the back of the animals about 24 h prior to study. Be careful not to injure the skin and only use healthy animals with undamaged skin.
2. When the test substance is a solid, grind it, as appropriate, and moisten it with water or other vehicle, so that it makes good contact with the skin. If using a vehicle, use one that will not irritate the skin, and be careful that the vehicle does not affect its permeability by the test substance. In general, use liquid test substance undiluted.
3. Apply locally 0.5 mL of liquid test substance, or 0.5 g of solid test substance in the form of a paste.

TABLE 3.6 (continued)
Comparison of Excerpts from Selected Dermal Irritation Test Guidelines

Japanese-MAFF

4. Apply the test substance to a small area of skin (approximately 6 cm²). During the administration (application) period, cover the site with a gauze patch, secured with a nonirritating tape. Procedures whereby the test substance, in the form of a liquid or paste, is applied to the gauze patch, which is then applied to the skin, are also acceptable. Using a semioclusive dressing, situate the patch in such a way that it will maintain contact with the skin throughout the exposure period (in some cases, an occlusive dressing may be used instead). Compare with the untreated areas of the animal's body.
5. Normally, the exposure period should be 4 h in duration. Use water or an appropriate vehicle to remove the test substance that is adhering to the skin at the end of the exposure period.
5. *Points to keep in mind regarding application*
 1. When severe skin irritant properties or corrosiveness are suspected
 - i. Conduct the test with only one animal.
 - ii. When corrosiveness is suspected, apply three test patches simultaneously to one animal. The first patch is removed after 3 min. If no strong skin reaction is observed, the second patch is removed after 1 h. If it is judged in terms of the humane treatment of the animal at this stage, then the exposure can be extended to 4 h, the third patch is removed after 4 h, and the reactions are graded. If a strong irritant reaction is observed after 3 min or 1 h exposure, the remaining patches are removed and the test stopped immediately. The three patches may also be applied successively to different sites on the same animal for examination.
 - iii. When it is suspected that the test substance is a severe skin irritant, apply one patch to one animal for 4 h.
 - iv. If no severe irritation is observed after a 4 h exposure, two more animals are tested with one patch each for 4 h.
 2. If it is anticipated that severe irritation/corrosion will not occur with the test substance, the test is started using three animals, and one patch is applied to each for 4 h.
6. *Observation and rating of general condition*

The animals are examined and scored for signs of erythema and edema 30 or 60 min, and 24, 48, and 72 h after removal of the patch. Skin irritation is scored and recorded in accordance with the evaluation scores given in the appendix. If necessary, subsequent examinations are given to demonstrate reversibility. In general, there is generally no need to go beyond 14 days. In addition to examining for irritation, any serious injury or other toxic reactions are recorded thoroughly.

European OECD (and European EEC)

Initial considerations

3. In the interest of both sound science and animal welfare, *in vivo* testing should not be undertaken until all available data relevant to the potential dermal corrosivity/irritation of the substance have been evaluated in a weight-of-the-evidence analysis. Such data will include evidence from existing studies in humans and/or laboratory animals, evidence of corrosivity/irritation of one or more structurally related substances or mixtures of such substances, data demonstrating strong acidity or alkalinity of the substance (2),(3), and results from validated and accepted *in vitro* or *ex vivo* tests (4)–(6). This analysis should decrease the need for *in vivo* testing for dermal corrosivity/irritation of substances for which sufficient evidence already exists from the other studies as to those two endpoints.
4. A preferred sequential testing strategy, which includes the performance of validated and accepted *in vitro* or *ex vivo* tests for corrosion/irritation, is included as a Supplement to this Guideline. The strategy was developed at, and unanimously recommended by participants of, an OECD workshop (7), and has been adopted as the recommended testing strategy in the Globally Harmonized System for the Classification of Chemical Substances (GHS) (8). It is recommended that this testing strategy be followed prior to undertaking *in vivo* testing. For new substances, it is the recommended stepwise testing approach for developing scientifically sound data on the corrosivity/irritation of the substance. For existing substances with insufficient data on dermal corrosion/irritation, the strategy should be used to fill missing data gaps. Major deviation from the testing strategy or procedure, or a decision not to use a stepwise testing approach, should be justified.
5. If a determination of corrosivity or irritation cannot be made using a weigh-of-the-evidence analysis, consistent with the sequential testing strategy, an *in vivo* test should be considered (see Supplement).

Principle of the *in vivo* test

6. The substance to be tested is applied in a single dose to the skin of one or more experimental animals, untreated skin areas of the test animal(s) serving as control. The degree of irritation is read and scored at specified intervals and is further described in order to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility of the effects observed.
7. Animals showing continuing signs of severe distress and/or pain at any stage of the test should be humanely killed, and the substance assessed accordingly.

(continued)

TABLE 3.6 (continued)
Comparison of Excerpts from Selected Dermal Irritation Test Guidelines

European OECD (and European EEC)

Selection of animal species

8. The albino rabbit is the preferable laboratory animal, and healthy young adult rabbits are used. A rationale for using another species should be provided.

Preparation of the animals

9. Approximately 24 h before the test, fur should be removed by close-clipping the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin, and only animals with healthy intact skin should be used.
10. Some strains of rabbit have dense patches of hair which are more prominent at certain times of the year. Such areas of dense hair growth should not be used as patch sites.

Housing and feeding conditions

11. Animals should be individually housed. The temperature of the experimental animal room should be 20°C ($\pm 3^\circ\text{C}$) for rabbits. Although relative humidity should be at least 30 and preferably not exceed 70%, other than during room cleaning, the aim should be 50%–60%. Lighting should be artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unrestricted supply of drinking water.

Procedure

Application of the test substance

12. The test substance should be applied to a small area (approximately 6 cm²) of skin and covered with a gauze patch, which is held in place with a nonirritating tape. In the case in which direct application is not possible (e.g., liquids or some pates), the test substance should be first applied to the gauze patch, which is then applied to the skin. The patch should be loosely held in contact with the skin by means of a suitable semioclusive dressing for the duration of the exposure period. If the test substance is applied to the patch, it should be attached to the skin in such a manner that there is good contact and uniform distribution of the substance on the skin. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.
13. Liquid test substances are generally used undiluted. When testing solids (which may be pulverized if considered necessary), the test substance should be moistened with the smallest amount of water (or where necessary, a suitable vehicle) sufficient to ensure good contact with the skin. When vehicles other than water are used, the potential influence of the vehicle on irritation of the skin by the test substance should be minimal if any.
14. At the end of the exposure period, which is normally 4 h, residual test substance should be removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Dose level

15. A dose of 0.5 mL of liquid or 0.5 g of solid or semisolid is applied to the test site.

Initial test (*in vivo* dermal irritation/corrosion test using one animal)

16. It is strongly recommended that the *in vivo* test be performed initially using one animal, especially when the substance is suspected to have corrosion potential.
17. When a substance has been judged to be corrosive on the basis of a weight-of-evidence analysis, no further animal testing is needed. For most substances suspected of being corrosive, further *in vivo* testing is normally not necessary. However, in those cases where additional data are felt warranted because of insufficient evidence, limited animal testing may be carried out using the following approach: Up to three test patches are applied sequentially to the animal. The first patch is removed after 3 min. If no serious skin reaction is observed, a second patch is applied at a different site and removed after 1 h. If the observations at this stage indicate that the exposure can humanely be allowed to extend to 4 h, a third patch is applied and removed after 4 h, and the response is graded.
18. If corrosive effect is observed after any of the three sequential exposures, the test is immediately terminated. If a corrosive effect is not observed after the last patch is removed, the animal is observed for 14 days, unless corrosion develops at an earlier time point.
19. In those cases in which the test substance is not expected to produce corrosion but may be irritating, a single patch should be applied to one animal for 4 h.

TABLE 3.6 (continued)
Comparison of Excerpts from Selected Dermal Irritation Test Guidelines

European OECD (and European EEC)

Confirmatory test (*in vivo* dermal irritation test with additional animals)

20. If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals, each with one patch, for an exposure period of 4 h. If an irritant effect is observed in the initial test, the confirmatory test may be conducted in a sequential manner, or by exposing two additional animals simultaneously. In the exceptional case, in which the initial test is not conducted, two or three animals may be treated with a single patch, which is removed after 4 h. When two animals are used, if both exhibit the same response, no further testing is needed. Otherwise, the third animal is also tested. Equivocal responses may need to be evaluated using additional animals.

Observation period

21. The duration of the observation period should not be fixed rigidly but should be sufficient to evaluate fully the reversibility of the effects observed. However, the experiment should be terminated at any time that the animals show continuing signs of severe pain or distress. To determine the reversibility of effects, the animals should be observed up to 14 days after removal of the patches. If reversibility is seen before 14 days, the experiment should be terminated at that time.

Clinical observations and grading of skin reactions

22. Animals should be examined for signs of erythema and edema, and the responses scored at 60 min, and then at 24, 48, and 72 h after patch removal. Dermal irritation is scored and recorded according to the grades in Table 3.7. For the initial test in one animal, the test site is also examined immediately after the patch has been removed. Dermal reactions are graded. If there is damage to the skin which cannot be identified as irritation or corrosion at 72 h, observations may be needed until day 14 to determine the reversibility of the effects. In addition to the irritation observations, all local toxic effects should be recorded. Histopathological examination should be considered to clarify equivocal responses.
23. The grading of skin responses is necessarily subjective. To promote harmonization in grading of skin response and to assist testing laboratories and those involved in making and interpreting the observations, the personnel performing the observations need to be adequately trained in the scoring system. An illustrated guide for grading skin irritation and other lesions could be helpful.

Data and reporting

24. Study results should be summarized in tabular form in the final test report and overall items listed in paragraph 27.

Evaluation of results

25. The dermal irritation scores should be evaluated in connection with the nature and severity of lesions, and their reversibility or lack of reversibility. The individual scores do not represent an absolute standard for the irritant properties of a material, as other effects of the test material are also evaluated. Instead, individual scores should be viewed as reference values, which need to be evaluated in combination with all other observations from the study.
26. Reversibility of dermal lesions should be considered in evaluating irritant responses. When responses such as alopecia (limited area), hyperkeratosis, hyperplasia, and scaling persist to the end of the 14-day observation period, the test substance should be considered an irritant.

Sources: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2500, Acute Dermal Irritation, August, 1998; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 404: Acute Dermal Irritation/Corrosion, 1, 2002; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.4. Acute Toxicity (Skin Irritation), 2008; Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Sensitization Studies (2-1-6), November 16–18, 2000.

TABLE 3.7
Comparison of Excerpts from Selected Sensitization Test Guidelines

EPA OPPTS 870.2600

Introduction

This guideline is one of the series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in the publications of the National Technical Information Service (NTIS), and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the US EPA under the Toxic Substance Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

(a) Scope

(1) *Applicability*: This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 USC 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 USC 2601).

(2) *Background*: The source materials used in developing this harmonized OPPTS test guidelines are the OPPTS Harmonized Test Guidelines Series 870, Guideline 870.2600 Skin Sensitization, dated August 1998; and OECD 406 Skin Sensitization (adopted July 1992); and the OECD 429 Skin Sensitization: LLNA (adopted April 2002).

(b) *Purpose*. The purpose of the selected test is to identify substances with skin sensitization potential. Determination of the potential to cause or elicit skin sensitization reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity. Information derived from skin sensitization tests serves to identify possible hazards to population exposed repeatedly to a test substance. Testing is not required if the test material is a known skin sensitizer. If it is suspected that the test material is a strong dermal irritant, see OPPTS 870.2000, paragraph (d)(2)(iii).

(c) Definitions.

Challenge exposure is an experimental exposure of a previously treated subject to a test substance following an induction period, to elicit a contact hypersensitivity response.

Induction exposure is the administration of a test substance to the test subject with the intention of inducing contact sensitization.

Induction period is a period of at least 1 week following an induction exposure during which sensitization may develop.

Skin sensitization (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In humans, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other species, the reactions may differ, and only erythema and edema may be seen.

Stimulation index (SI) is the ratio of ³H-methyl thymidine or ¹²⁵I-iododeoxyuridine (¹²⁵IU) incorporation into the test group lymph nodes relative to that recorded for solvent/vehicle control group lymph nodes.

(d) Test procedures

(1) *Methods*: Any of the following test methods is considered to be acceptable:

- (i) LLNA test
- (ii) BT
- (iii) GPMT

(2) *Choice of assays*: See OPPTS 870.1000 for a general discussion of factors to be considered prior to performing the test.

- (i) The tester should note that LLNA may not be appropriate for all types of test materials, such as certain metallic compounds, high-molecular-weight proteins, strong dermal irritants, and materials that do not sufficiently adhere to the ear for an acceptable period of time during treatment. When using the LLNA, particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles or test material and runny liquids are to be avoided. In all instances, the tester must document that appropriate techniques were used to facilitate adherence to the mouse ear of adequate exposure duration.
- (ii) In situations for test materials where LLNA is not applicable or may provide unreliable or problematic results, the GPMT or BTs are recommended.
- (iii) Although LLNA, GPMT or BTs are considered to be acceptable tests, it is recognized that other tests may give useful results. If other tests are used, the investigator must provide justification/reasoning for use of the other procedures, and methods and protocol must be provided. A positive and negative control group must be included in each test.

(e) Test methods

(1) LLNA method

(i) *Principle of the test methods*: The basic principle underlying the LLNA is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of application. *Details are listed within the guideline.*

(ii) Animal selection

(A) *Species and strain*: Young adult female mice (nulliparous and nonpregnant) of the CBA/Ca or CBA/J strain should be used at age 8–12 weeks. All animals are to be age-matched (preferably with a 1-week time frame). Females are used because the existing database is predominantly based on this gender. Males and other strains of mice should not be used until it is sufficiently demonstrated that the significant strain-specific and/or gender-specific differences in the LLNA response do not exist.

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

EPA OPPTS 870.2600

(B) *Housing and feeding*: The temperature of the experimental animal room should be $21^{\circ}\text{C} \pm 3^{\circ}\text{C}$ with the relative humidity of 30%–70%. Where the lighting is artificial, the sequence should be 12 h light: 12 h dark. For feeding, standard laboratory mouse diets are to be used with an unlimited supply of drinking water. The mice must be acclimatized for at least 5 days prior to the start of the test. Animals must be housed individually. Healthy animals are randomly assigned to the control and treatment groups having statistically homogenous body weights. Although a variety of techniques exist to uniquely mark mice, any method that involves identification via ear marking (e.g., ear tags) must not be used.

(iii) Test conditions

(A) *Preparation of doses*: Solid test substances are to be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Stable suspensions might also be acceptable. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance are to be prepared daily unless stability data demonstrate the acceptability of storage.

(B) *Solvent/vehicle*: The solvent/vehicle is to be selected on the basis of maximizing the test concentration while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used if appropriately justified. The selected vehicle must not interfere with or bias the test result and should be selected to achieve the maximum concentration/skin exposure of the test substance. Ensure that hydrophilic materials are incorporated into the vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles should be avoided.

(C) Controls

(1) Concurrent negative (solvent/vehicle) and positive controls are to be included in each test. In some circumstances, it may be useful to include a naïve control. Except for treatment with the test substance, animals in the control groups are to be handled in an identical manner to the animals of the treatment groups.

(2) Positive controls are used to ensure the appropriate performance of the assay. The positive control must produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) of 3 or greater over the solvent or vehicle control group. The positive control dose is to be chosen such that the induction is clear but not excessive. Preferred positive control substances are HCA and mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used. However, benzocaine should not be used as a positive control in LLNA.

(3) The positive control substance is tested in the vehicle that is known to elicit a consistent response (i.e., acetone/olive oil). If a nonstandard vehicle is used with a positive control, the nonstandard vehicle must be tested for a local lymph node response prior to the initiation of the study and results reported.

(iv) LLNA test procedure

(A) Details are listed within the guideline

(v) Data interpretation and reporting for LLNA

(A) Data interpretation.

(1) A substance is regarded as a skin sensitizer in the LLNA if at least one concentration of the test material results in a threefold or greater increase in 3H-methyl thymidine or 125IU incorporation in the lymph node cells of test group lymph nodes relative to that recorded for solvent/vehicle control lymph nodes, as indicated by the SI. However, the magnitude of the SI should not be the sole factor in determining the biological significance of a skin sensitization response. A quantitative assessment must be performed by statistical analysis of individual animal data in order to provide a more completed evaluation of the test substance. Factors to be considered in evaluating the biological significance of a response or outcome of the test include the results of the SI determination, statistical analyses, the strength of the dose–response relationship, chemical toxicity, solubility, and the consistency of the solvent/vehicle and positive control responses.

(2) Strong irritants may yield false-positive resulting in the LLNA due the initiation of a significant lymphocyte proliferation. However, the dose–response information from the assay may help to uncover a strong irritant response since, for instance, it has been shown that the proliferation induced by irritation usually results in a shallow dose–response relationship. Concurrent evaluation of ear swelling may also provide helpful information on differentiating weak sensitizers from strong irritants.

(2) GPMT and Buehler methods

(i) *Principle of the test methods*: Following initial exposure to a test substance, the animals are subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish where a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction with that of initial induction exposure. The test animals are initially exposed to the test substance by intradermal and/or epidermal application (induction exposure). Following a rest period of 10–14 days (the induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of the skin reaction to the challenge exposure are compared with that demonstrated by control animals that undergo sham treatment during induction and then receive the challenge exposure.

(ii) Animal selection

(A) *Species and strain*: The young adult guinea pig is the preferred species. Young adult commonly used laboratory strains must be employed.

(B) *Housing and feeding*: The temperature of the experimental animal room should be $20^{\circ}\text{C} \pm 3^{\circ}\text{C}$ with the relative humidity of 30%–70%. Where the lighting is artificial, the sequence should be 12 h light/12 h dark. Conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

(continued)

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

EPA OPPTS 870.2600

- (C) *Number and sex*: The number and sex will depend on the method used. Either sex may be used in the BT and the GPMT. If females are used, they should be nulliparous and not pregnant. The BT recommends using a minimum of 20 animals in the treatment and at least 10 as controls. At least 10 animals in the treatment group and 5 in the control group must be used in the GPMT, with the stipulation that if it is not possible to conclude that the test substance is a sensitizer after using fewer than 20 test and 10 control guinea pigs, the testing of additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.
- (D) *Control animals*
- (2) Every 6 months, assess the sensitivity and reliability of the experimental technique in naive animals by the use of positive control substance known to have mild-to-moderate skin-sensitizing properties. In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a nonadjuvant test should be expected for mild-to-moderate sensitizers. Preferred substances are HCA (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4), benzocaine (CAS No. 94-09-7), DNCB (CAS No. 97-00-7), or DER 331 epoxy resin. There may be circumstances where, given adequate justification, other control substances meeting the mentioned criteria may be used.
- (E) *Dose levels*: The dose level will depend on the test method selected. In BT, select the concentration of the induction dose such that it is high enough to cause mild irritation and the challenge dose such that it is the highest nonirritating concentration. In GPMT, the concentration of the induction dose must be well tolerated systemically, and must be high enough to cause mild-to-moderate skin irritation; the GPMT challenge dose must use the highest nonirritation concentration.
- (F) *Observation of animals*
- (1) Skin reactions are to be graded and recorded after the challenge exposures at the time specified by the methodology selected. This is usually at 24 and 48 h. Additional notations should be made as necessary to fully describe unusual responses.
- (2) Regardless of the method selected, initial and terminal body weights must be taken and recorded.
- (G) *Procedures*: The procedures to be used are those described by the test method chosen.

Details are listed within the guidelines.

Japanese-MHW

2. Selection of test methods

These test methods are given as examples because they have been adopted in most laboratories, and because that they all represent well-established assay techniques with a high degree of reproducibility. Generally, original reports pertaining to the individual test methods are cited herein, although some of these tests are in use with modifications. Testing procedure need not be limited to those cited herein, and in cases where any other test method is employed, justification of its application should be stated along with citation of the appropriate literature.

(a) **Adjuvant and patch test**

The test comprises intradermal injections of FCA and abrasion of the skin, topical application of the test substance onto the scratched region, and covering of the test site with an occlusive patch for sensitization. The topical challenge is made without a covering. The test is used for such test substances which are not injectable intradermally.

(b) **Buehler test**

This test also employs topical application of the test substance. The test site is covered with an occlusive patch and a wrapping, and the topical challenge is carried out with a wrap, as during induction, to enhance penetration and prevent evaporation of the test substance.

(c) **Draize test**

The test method was the first predictive sensitization assay accepted by regulatory agencies. It is characterized by the intradermal introduction of a dilution of the test substance for sensitization, and a challenge by subsequent intradermal injection.

(d) **FCA test**

The test comprises intradermal injections of the test substance incorporated in a 1:1 mixture of FCA and distilled water.

(e) **Maximization test**

The maximization test, as described in the Guidelines, combines FCA, SLS, intradermal injection, and occlusive topical application of the test substance during the sensitization period.

(f) **Open epicutaneous test**

This test closely simulates the conditions of drug use in humans by utilizing repeated topical application of the test substance.

(g) **Optimization test**

The optimization test is analogous to Draize test, but involves the use of FCA for sensitization and an intradermal challenge with covering of the test site.

(h) **Split adjuvant test**

The test utilizes skin damage caused by the application of dry ice onto a shaved area of skin, and FCA as an adjuvant. The test substance is applied topically with a dressing.

Whichever test method is selected, it is impracticable to accomplish a perfect prediction of the sensitizing potential of a substance in humans based solely on results of the test, but the test results may provide important information valid for the extrapolation of data to the conditions of human use. In the current Guidelines, the spirit of concern for research animals is incorporated. Test methods, for example, are roughly divided into two groups: those involving the use of an adjuvant and those which do not, so as to systematically minimize the types of tests to be adopted. Thus, scientific considerations have been made for the reduction of the number of animals used in tests. They are, in brief, (1) reduction of the number of animals used in tests, (2) limitation of types of testing methods and delineation of test methods that may be selected, (3) classification of the test methods according to assay sensitivity, and so forth.

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

Japanese-MHW

3. Selection of test animals

Primarily, animals highly susceptible to the sensitizing action of the test substance are to be selected as a test system. In all the test methods mentioned earlier, regardless, the animal species used is the guinea pig. Young, healthy adult albino guinea pigs (usually between 1 and 3 months of age) weighing not more than 500 g at the start of the test are used as a rule. They may be male or female, or of both sexes, and, in the case of females, the animals should be nonpregnant and nulliparous.

This animal species is selected primarily for the reasons that guinea pigs are known to elicit, if at all, reactions similar to those that occur in man and that a substantial amount of background laboratory data has been accumulated for this species.

4. Number of animals

An extreme reduction in the number of animals used in tests may render statistical data analysis meaningless. Only the minimum number of animals required for test groups (groups subject to sensitization with the test substance) and control groups (positive control and control groups) are stated in these Guidelines. If any influence of the minimized number of animals on test results is anticipated, the number should be increased appropriately. The earlier stated minimum number of animals (five/group) may suffice only for such circumstances where the response is either obviously negative or strongly positive. It follows that, otherwise, each test group need to consist of at least 10 animals and each control group of at least 5 animals.

5. Positive controls

Positive controls are required as references for comparative assessments of the responsiveness of animals used and of the sensitizing potency of the drug substance being tested. Compounds currently in use for this purpose include: *p*-phenylenediamine (CAS No. 106503), 1-chloro-2,4-dinitrobenzene (CAS No. 97007), neomycin sulfate (CAS No. 1405103), and nickel sulfate (CAS No. 7786814), but any other suitable sensitizers documented in the biomedical literature may also be used.

6. Test methods

Detailed accounts are given of the maximization test (Magnusson and Kligman) and the adjuvant and patch test, under Description of the Test Procedure in the Guidelines, while the other six test methods are only cited. All the test methods mentioned in the Guidelines may be regarded as essentially equivalent and none given a preference to others. That is, any of the test methods mentioned earlier may be adopted.

The maximization test and adjuvant and patch test are selected as examples to be detailed on the rationale that those involving the use of FCA are likely to be superior in assay sensitivity to those not using it.

It is most desirable to conduct the testing stepwise in evaluating a substance for skin sensitizing potential. In the first step, one of the five tests incorporating the use of the adjuvant is to be performed to ascertain if the property of the test substance is to be further assessed by comparison with a known sensitizing substance or by conducting a test not involving the use of an adjuvant so as to permit evaluation of the intensity of sensitization by the test substance.

All these tests are designed to determine the potential of test substances to induce hypersensitivity by, in general, exposure (sensitization) of experimental animals to the test substance and a challenge exposure (elicitation) after a subsequent rest period of about 2 weeks. Test results are for sensitizing potential interpreted by comparing cutaneous responses of experimental animals with those of controls. Each of the test methods described has advantages and drawbacks and, therefore, it is most desirable that the tests be performed properly by personnel well-versed in these aspects.

In preparing animals for tests, they should be randomly allocated to experimental groups. Test sites on the skin must be clipped free of hair or shaven prior to administration of the test substance.

Study conditions must be carefully set up, since test results may vary with the conditions.

7. Dose levels

In case where graded dose levels appropriate to the assay are employed, the physicochemical properties of the test substance, such as the solubility at test concentrations and the tolerated local or systemic dose need be taken into account. The concentrations of the test substance for sensitizing and challenge chosen for the assay may be justified whenever deemed necessary.

8. Observation parameters

Body weights of animals must be recorded at least at the initiation and at the completion of testing. All animals should be observed for any signs of skin irritation during the sensitization period. Skin reactions are to be noted at 24, 48, or 72 h after challenge exposure, and interpreted for sensitizing activity of the test substance. The reactions must be rated as specified for each test. All observed cutaneous reactions and any adverse findings noted must be recorded.

9. Reporting of test results

It is advisable that test results be summarized by tabulation or other means in such a way that the skin reactions of individual animals at respective periods of observation can be clearly recognized.

In reporting the test results, data concerning the following parameters must be included:

- (1) Strain of guinea pigs used.
- (2) Number, age in weeks, and sex(es) of animals used.
- (3) Individual animal body weights at the start and at the completion of test.
- (4) All reactions observed in animals, along with details of reactions if any scoring system or classification scheme is employed.
- (5) Evaluation of test results, and comments.

10. Evaluation of test results

The skin-sensitizing potential of the test substance should be evaluated according to the reactions observed in animals in the test group and in each control group. Interpretation of the test results must be based on evaluation of the potential of the test substance to sensitize the skin. Basically, it is to be made according to the evaluation criteria specified in the literature reporting the test method.

(continued)

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

Japanese-MHW

In cases where the incidence of a positive skin reaction is to be assessed, it is advisable that increased numbers of animals be used in test and control groups, and that data obtained be processed by an appropriate statistical procedure.

What should be noted here is that the tests mentioned herein, unfortunately, are not necessarily adequate as assays for predicting the sensitizing potential of the test substance in humans. To evaluate the sensitizing activity of the material, therefore, the material is first to be subjected to any one of the test methods involving the use of an adjuvant and determined thereby as to whether it has sensitizing activity or not. If the material has proven to be positive, then it should be further assessed, preferably by a test method not involving the use of an adjuvant in order to make practical risk assessment and classification of the test substance.

Japanese-MAFF

- 1. Objective
The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding skin sensitization by the test substance.
- 2. Test animal species, age, and sex
 - (1) Use young adult guinea pigs.
 - (2) Nulliparous, nonpregnant females should be used.
- 3. Study methods
The GPMT (GPM method) and the BT (Buehler method) are study methods that are conducted relatively frequently. However, other methods may be substituted if information regarding sensitization can be obtained thereby.
- 4. Study procedures
GPM method and the Buehler method are described in detail in the guidelines.
 - (1) *Number of animals*: This is dependent on the method used.
 - (2) *Dose settings*: The maximum concentration of the test substance used in exposure for sensitization is one to which there is satisfactory resistance systemically but producing mild-to-moderate skin irritation. The maximum concentration of test substance used for challenge exposure is the highest at which no irritation is shown. Two or three animals are used to determine the appropriate concentration of the test substance.
 - (3) *Sensitization*: Procedures are dependent on the method used. Details are listed within the Guidelines. Ventral patch application is referenced.
 - (6) *Examination*: About 21 h after removing the patches, the challenged area is shaved if necessary. Three hours later (about 48 h after the start of application of the challenging patches), the skin is examined for any reaction which is then recorded in accordance with the grades shown in the following table. Skin reactions are observed and recorded again 24 h after the first examination.

Table Evaluation Criteria for Challenge Patch Test Reaction

No visible change	0
Diffuse or patchy erythema	1
Moderate dispersed erythema	2
Severe erythema and edema	3

- (8) Examination of general condition. All skin reactions and all abnormal findings which occur as a result of sensitization and challenge are recorded.

European OECD 406 Skin Sensitization

- Introduction
- 2. Currently, quantitative structure–activity relationships and *in vitro* models are not yet sufficiently developed to play a significant role in the assessment of the skin-sensitization potential of substances which therefore must continue to be based on *in vitro* models.
 - 3. The guinea pig has been the animal of choice for predictive sensitization tests for several decades. Two types of tests have been developed: adjuvant tests in which sensitization is potentiated by injection of FCA, and nonadjuvant tests. In the original guideline 406, four adjuvant tests and three nonadjuvant tests were considered to be acceptable. In this updated version, the GPMT of Magnusson and Kligman which uses adjuvant and the nonadjuvant BT are given preference over other methods, and the procedures are presented in detail. It is recognized, however, that there may be circumstances where other methods may be used to provide the necessary information on sensitization potential.
 - 4. The immune system of the mouse has been investigated more extensively than that of the guinea pig. Recently, mouse models for assessing sensitization potential have been developed that offer the advantages of having an end point which is measured objectively, being of short duration, and treating a minimal number of animals. The mouse ear swelling test (MEST) and the LLNA appear to be promising. Both assays have undergone validation in several laboratories and it has been shown that they are able to detect reliably moderate to strong sensitizers. The LLNA or the MEST can be used as a first stage in the assessment of skin sensitization potential. If a positive result is seen in either assay, a test substance may be designated as a potential sensitizer, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in the LLNA or MEST, a guinea pig test (preferably a GPMT or BT) must be conducted using the procedure described in this guideline.
 - 5. Definitions:
 - Skin sensitization* (allergic contact dermatitis): An immunologically mediated cutaneous reaction to a substance. In the human, pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these may characterize the responses. In other species, the reactions may differ and only erythema and edema may be seen.
 - Induction exposure*: An experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.
 - Induction period*: A period of at least 1 week following an induction exposure during which a hypersensitive state may develop.
 - Challenge exposure*: An experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

European OECD 406 Skin Sensitization

General principle of sensitization tests in Guinea pigs

6. The test animals are initially exposed to the test substance by intradermal injection and/or epidermal application (induction exposure). Following a rest period of 10–14 days (induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals are compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

Elements common to sensitization tests in Guinea pigs

Sex of Animals

7. Male and/or female healthy young adult animals can be used. If females are used, they should be nulliparous and nonpregnant.

Housing and feeding conditions

8. The temperature of the experimental animal room should be 20°C (±3°C) and the relative humidity 30%–70%. Where the lighting is artificial, the sequence should be 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

Preparation of the animals

9. Animals are acclimatized to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomized and assigned to the treatment groups. Removal of hair is by clipping, shaving, or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

Reliability check

10. The sensitivity and reliability of the experimental technique used should be assessed every 6 months by use of substances which are known to have mild-to-moderate skin sensitization properties.
11. In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a nonadjuvant test should be expected for mild/moderate sensitizers. Preferred substances are HCA (CAS No. 101860), mercaptobenzothiazole (CAS No. 149304), and benzocaine (CAS No. 94097). There may be circumstances where, given adequate justification, other control substances meeting the earlier criteria may be used.

Removal of the test substance

12. If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Description of the Guinea pig methods

- 13–36. Both the GPMT and BT methods are described in the guidelines.

European EEC B.6 Skin Sensitization

Method

1.1 Introduction

Remarks:

The sensitivity and ability of tests to detect potential human skin sensitizers are considered important in a classification system for toxicity relevant to public health. There is no single test method which will adequately identify all substances with a potential for sensitizing human skin and which is relevant for all substances.

Factors such as the physical characteristics of a substance, including its ability to penetrate the skin, must be considered in the selection of a test.

Two types of tests using guinea pigs can be subdivided into the adjuvant-type tests, in which an allergic state is potentiated by dissolving or suspending the test substance in FCA, and the nonadjuvant tests.

Adjuvant-type tests are likely to be more accurate in predicting a probable skin-sensitizing effect of a substance in humans than those methods not employing FCA and are thus the preferred methods.

The GPMT is a widely used adjuvant-type test. Although several other methods can be used to detect the potential of a substance to provoke skin-sensitization reaction, the GPMT is considered to be the preferred adjuvant technique.

With many chemical classes, nonadjuvant tests (the preferred one being the BT) are considered to be less sensitive.

In certain cases, there may be good reasons for choosing the BT involving topical application rather than the intradermal injection used in the GPMT. Scientific justification should be given when the BT is used.

The GPMT and BT are described in this method. Other methods may be used provided that they are well-validated and scientific justification is given. If a positive result is seen in a recognized screening test, a test substance may be designated as a potential sensitizer, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in such a test, the guinea pig test must be conducted using the procedure described in the test method.

1.3 Reference substances

The sensitivity and reliability of the experimental technique used should be assessed every 6 months by use of substances, which are known to have mild-to-moderate skin-sensitization properties.

In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a nonadjuvant test should be expected for mild/moderate sensitizers. The following substances are preferred.

Cas number 101-86-0; α -HCA

Cas number 149-30-4; Benzothiazole-2-thiol (mercaptobenzothiazole)

(continued)

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

European EEC B.6 Skin Sensitization

Cas number 94-09-7; Benzocaine

1.4–1.5.2.3.3. Both the GPMT and the BT methods are described in the guidelines.

European OECD 429/442A/442B Skin Sensitization:LLNA and European EEC B.42 Skin Sensitization:LLNA

Initial considerations and limitations

4. The LLNA provides an alternative method for identifying potential skin-sensitizing test substances. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig tests, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation. The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the test substance; its physicochemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the test substance; and toxicological data on structurally related test substances. This information should be considered in order to determine whether the LLNA, LLNA:DA or LLNA is appropriate for the test substance and to aid in dose selection.
5. A synopsis of advantages and limitations are listed as follows (See appropriate guidelines for specific information):

Advantages of LLNA:

- Does not require that challenge-induced dermal hypersensitivity reactions be elicited
- Does not require the use of an adjuvant as used in the GPMT
- Provides quantitative data for possible dose-response relationships (Stimulation Indices)
- Potential to reduce the overall number of animals utilized compared to other assays

Limitations of LLNA that may necessitate guinea pig tests:

- Possible false-positive findings with certain skin irritation (e.g., surfactants)
- Possible false-negative findings with certain metals
- Solubility of the test substance (wholly aqueous materials are not advised)
- Test substance classes or substances containing functional groups shown to act as potential confounders
- More likely to yield a positive result for pesticide substances (OECD 429 methodology)
- Not appropriate for test substances that affect ATP levels (ATP inhibitors) or accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node under OECD 442A methodology)

6. Principle of the tests

The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of test substance application.

This proliferation is proportional to the dose and the potency of the applied allergens and provides a simple means of obtaining a quantitative measurement of sensitization. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control (VC) group. The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the Stimulation Index (SI) is determined and should be SI > 3 (OECD 429 incorporation by radiolabeled substance), SI > 1.6 (OECD 442A quantifies ATP content via bioluminescence as an indicator of lymphocyte proliferation; SI > 1.8 and < 2.5 borderline positive), or SI > 1.8 (OECD 442B lymphocyte proliferation is measured by ELISA utilizing an antibody specific for BrdU that is labeled with peroxidase and reacts with a substrate; SI > 1.6 and < 1.9 borderline positive result) before clarification of the test substance as a potential skin sensitizer is warranted.

Elements Common to LLNA Tests in Mice

Selection of animal species

7. The mouse is the species of choice for this test. For OECD 429, young adult female mice of CBA/Ca or CBA/J strain are used. For OECD 442A, validation studies for the LLNA: DA were conducted exclusively with the CBA/J strain, which is therefore the preferred strain. For OECD 442B, validation studies for the LLNA: BrdU-ELISA were conducted exclusively with the CBA/JN strain, which is therefore considered the preferred strain. Young adult female mice, which are nulliparous and nonpregnant, are used. At the start of the study, animals should be between 8 and 12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. Alternatively, other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA BrdU-ELISA response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (16), unless adequate scientific rationale for housing mice individually is provided. The temperature of the experimental animal room should be 22°C ± 3°C. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50%–60%. Lighting should be artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Preparation of animals

9. The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatization to the laboratory conditions. Prior to the start of treatment, all animals are examined to ensure that they have no observable skin lesions.

Preparation of dosing solutions

10. Solid test substances should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to the ear of the mice. Liquid test substances may be applied neat or diluted prior to dosing. Insoluble substances, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to application to the ear of the mice. Test substances should be prepared daily unless stability data demonstrate the acceptability of storage.

TABLE 3.7 (continued)
Comparison of Excerpts from Selected Sensitization Test Guidelines

Elements Common to LLNA Tests in Mice

Reliability check

11. Positive controls are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing test substance for which the magnitude of the response is well characterized. Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra-competency of the laboratory to successfully conduct each assay and allows for an assessment of intra-and inter-laboratory reproducibility and comparability. Some regulatory authorities also require a PC for each study and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA of choice. Accordingly, the routine use of a concurrent PC is encouraged to avoid the need for additional animal testing to meet such requirements that might arise from the use of a periodic PC (see paragraph 12). The PC should produce a positive LLN response at an exposure level (OECD 429 LLNA should produce an increase in SI > 3 over the negative control group; OECD 442A LLNA:DA would give an increase in SI \geq 1.8 over the negative control; OECD442B LLNA: BrdU-ELISA would give an increase in the SI \geq 1.6 over the negative control group). The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (e.g., for OECD 429 LLNA, an SI > 20 would be excessive; for OECD 442A LLNA:DA, an SI > 10 would be considered excessive; for OECD 442B LLNA: BrdU-ELISA, SI > 14 would be considered excessive). Preferred PC test substances are: LLNA (OECD429), LLNA:DA, and LLNA: BrdU-ELISA is 25% HCA and 25% eugenol in acetone: olive oil (4:1, v/v) and LLNA (OECD429) 5% mercaptobenzothiazole in N,N-dimethylformamide. There may be circumstances in which, given adequate justification, other PC test substances, meeting the previous criteria, may be used.
12. While inclusion of a concurrent PC group is recommended, there may be situations in which periodic testing (i.e., at intervals \leq 6 months) of the PC test substance may be adequate for laboratories that conduct the LLNA regularly (LLNA: BrdU-ELISA at a frequency of no less than once per month) and have an established historical PC database that demonstrates the laboratory's ability to obtain reproducible and accurate results with PCs. Adequate proficiency with LLNA can be successfully demonstrated by generating consistent positive results with PC in at least 10 independent tests conducted with a reasonable period of time (i.e., <1 year).
13. A concurrent PC group should always be included when there is a procedural change to the LLNA (e.g., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the PC results.
14. Investigators should be aware that the decision to conduct a PC study on periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent PC during the interval between each periodic PC study. For example, if a false-negative result is obtained in the periodic PC study, negative test substance results obtained in the interval between the last acceptable periodic PC study and the unacceptable periodic PC study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent PC group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used.
15. Although the PC test substance should be tested in the vehicle that is known to elicit a consistent response (e.g., acetone: olive; 4:1, v/v), there may be certain regulatory situations in which testing in a nonstandard vehicle (clinically/chemically relevant formulation) will also be necessary. If the concurrent PC test substance is tested in a different vehicle than the test substance, then a separate VC for the concurrent PC should be included.
16. In instances where test substances of a specific chemical class or range of responses are being evaluated, benchmark test substances may also be useful to demonstrate that the test method is functioning properly for detecting the skin-sensitization potential of these types of test substances. Appropriate benchmark test substances should have the following properties:
 - Structural and functional similarity of the class of the test substance being tested
 - Known physical/chemical characteristics
 - Supporting data from the appropriate/same LLNA
 - Supporting data from other animal models and/or from humans
- 17–31. (See guidelines for test methodologies and further information).
32. For borderline positive responses, users may want to consider additional information such as dose–response relationship, evidence of systemic toxicity, or excessive irritation, and where appropriate, statistical significance together with SI values to confirm that such results are positive. Consideration should be given to various properties of the test substance including whether it has structural relationship to known skin sensitizer, whether it causes excessive skin irritation in the mouse, and the nature of the dose–response observed. SI > 20 (OECD429), SI > 10 (OECD442A), SI > 14 (OECD442B) would be considered excessive.
- 33–35. (See guidelines for test methodologies and further information).

Sources: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2600: Skin Sensitization Study, 2003; Japan New Drugs Division Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, 1990 Guidelines for Toxicity Studies of Drugs Manual, 1991, Chapter 7; Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Sensitization Studies (2-1-6), November 16–18, 2000; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 406: Skin Sensitization, 1992; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.6: Skin Sensitization, 2008; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 429: Skin Sensitization: Local Lymph Node Assay, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442A: Skin Sensitization: Local Lymph Node Assay: DA, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, 2010; The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.42: Skin Sensitization: Local Lymph Node Assay, 2008; U.S. Environmental Protection Agency, Office of Pesticide Programs: Expansion of the Traditional Local Lymph Node Assay for the Assessment of Dermal Sensitization Potential of End Use Pesticide Products and Adoption of a “Reduced” Protocol for the Traditional LLNA (Limit Dose), January 27, 2011.

MATERIALS AND PROCEDURES FOR PERFORMING DERMAL IRRITATION STUDIES²⁸⁻³⁷

OCCLUDED DERMAL IRRITATION TEST IN RABBITS

Materials

Gauze Dressing

Type: Ace-Tex Corporation nonsterilized
Thickness: 4-ply gauze dressing
Size: 1 × 1 in.

Tape—Nonirritating

Type: Blenderm® (Medical Surgical Division/3M, St. Paul, MN)
Size: 1 in. wide

Occlusive Materials

Type: Impervious material (e.g., plastic wrap)

Binding Materials

Elastic wrap

Type: Rubber elastic bandage: Ace® Bandage or Coban® (Medical Surgical Division/3M, St. Paul, MN), or Expandover® (Kendall Brand/Covidien, Mansfield, MA) and Ultra-light (Kendall Brand from Covidien, Mansfield, MA)

Size: Adequate wrapping of the entire test site

Securing Materials

Type: Zonas® porous athletic tape (Johnson & Johnson Medical Inc., Arlington, TX)
Size: 2 in. wide

Elizabethan or Similar Collars

Optional

Animal Species

New Zealand White Rabbits

Procedure

The hair is removed from a sufficient area on the rabbit's back on the day before dosing using a No. 40 clipper blade. Care should be taken to avoid abrading the skin during the clipping procedure. On the day of dosing, the test site (approximately 1 × 1 in.² of intact skin) should be designated with an indelible marker, and the gauze patch (1 × 1 in.) should be secured to the animal on at least two cut edges of the gauze patch, using the nonirritating Blenderm tape. The test substance, either 0.5 mL or 0.5 g, should be administered under the gauze dressing, and the remaining cut edges secured to the animal's back with a nonirritating tape. Liquids are administered as received; powders should be moistened with a suitable vehicle before application (e.g., distilled water). If the test article is a solid or powder that does not work well as a paste (e.g., does not spread well), 0.5 g of the test article will be applied to an approximate 1 × 1 in. 4-ply gauze patch and will be moistened with the appropriate amount of distilled water or suitable vehicle (generally 0.5 mL), and the gauze

patch applied to the test site. An impervious sheet of material (e.g., plastic wrap) is then wrapped around the trunk of the animal. The elastic wrap is then wrapped around the animal's torso and is secured in place using the Zonas athletic tape. The Zonas is wrapped around the outermost portion of the elastic wrap at the cranial and caudal ends. An Elizabethan or similar collar may then be placed around the animal's neck. After the designated time of exposure (i.e., 4 or 24 h), the tape, elastic wrap, impervious wrap, and gauze patch are removed, and the test site is delineated using an indelible marker. The test site should then be rinsed with a suitable vehicle (e.g., distilled water). At the appropriate grading intervals (e.g., 1, 24, 48, and 72 h after patch removal), the animal should be examined and scored for signs of erythema and edema according to the Draize dermal grading system. Grading of the test sites may be continued after the 72 h scoring interval if irritation persists (e.g., day 7, day 10, day 14).

SEMI-OCCLUDED DERMAL IRRITATION TEST IN RABBITS

Materials

Gauze Dressing

Type: Ace-Tex Corporation nonsterilized
Thickness: 4-ply gauze dressing
Size: 1 × 1 in.

Tape—Nonirritating

Type: Blenderm® (Medical Surgical Division/3M, St. Paul, MN)
Size: 1 in. wide

Binding Materials

Elastic wrap

Type: Rubber elastic bandage: Ace® Bandage, Coban® (Medical Surgical Division/3M, St. Paul, MN), or Albahealth® Stockinette (Encompass Group, McDonough, GA)

Size: Adequate wrapping of the entire test site

Securing Materials

Type: Zonas® porous athletic tape (Johnson & Johnson Medical Inc., Arlington, TX)
Size: 2 in. wide

Elizabethan or Similar Collars

Optional

Animal Species

New Zealand White Rabbit

Procedure

The hair is removed from a sufficient area on the rabbit's back on the day before dosing using a No. 40 clipper blade. Care should be taken to avoid abrading the skin during the clipping procedure. On the day of dosing, the test site (approximately 1 × 1 in.² of intact skin) should be

designated with an indelible marker, and the gauze patch (1 × 1 in.) should be secured to the animal on at least two cut edges of the gauze patch using the nonirritating Blenderm tape. The test substance, either 0.5 mL or 0.5 g, should be administered under the gauze dressing, and the remaining cut edges secured to the animal's back with a nonirritating tape. Liquids are administered as received. Powders should be moistened with a suitable vehicle prior to application (e.g., distilled water). If the test article is a solid or powder that does not work well as a paste (e.g., does not spread well), 0.5 g of the test article will be applied to an approximate 1 × 1 in. 4-ply gauze patch and will be moistened with the appropriate amount of distilled water or suitable vehicle (generally 0.5 mL), and the gauze patch applied to the test site. The elastic wrap is then wrapped around the animal's torso and secured in place using the Zonas athletic tape. The Zonas tape is wrapped around the outermost portion of the elastic wrap at the cranial and caudal ends. An Elizabethan or similar collar may then be placed around the animal's neck. After the designated exposure time (i.e., 4 or 24 h), the tape, elastic wrap, and gauze patch are removed, and the test site is delineated using an indelible marker. The test site should then be rinsed with a suitable vehicle (e.g., distilled water). At the appropriate grading intervals (e.g., 1, 24, 48, and 72 h after patch removal), the animal should be examined and scored for signs of erythema and edema according to the Draize dermal grading system. Grading of the test sites may be continued after the 72 h scoring interval if irritation persists (e.g., day 7, day 10, day 14).

NONOCCCLUDED DERMAL IRRITATION TEST IN RABBITS

Materials

Elizabethan or Similar Collars or Restrainer

Animal Species

New Zealand White Rabbit

Procedure

The hair is removed from a sufficient area on the rabbit's back on the day before dosing using a No. 40 clipper blade. Care should be taken to avoid abrading the skin during the clipping procedure. On the day of dosing, the test site (approximately 1 × 1 in.² of intact skin) should be designated with an indelible marker, and the test substance (0.5 mL or 0.5 g) should be administered to that area. Liquids are administered as received; powders should be moistened with a suitable vehicle before application (e.g., distilled water). The animal should then be placed in a restraining device, or an Elizabethan or similar collar should be applied. After the designated exposure time (i.e., 4 or 24 h), the test site should be delineated with an indelible marker and rinsed with a suitable vehicle (e.g., distilled water). At the appropriate grading intervals (e.g., 1, 24, 48, and 72 h following patch removal), the animal should be examined and scored for signs of

erythema and edema according to the Draize dermal grading system. Grading of the test sites may be continued after the 72 h scoring interval if irritation persists (e.g., day 7, day 10, and day 14).

DERMAL IRRITATION/CORROSIVITY TEST IN RABBITS

Materials

Gauze Dressing

Type: Ace-Tex Corporation nonsterilized

Thickness: 4-ply gauze dressing

Size: 1 × 1 in.

Tape Nonirritating

Type: Blenderm® (Medical Surgical Division/3M, St. Paul, MN)

Size: 1 in. wide

Binding Materials

Elastic wrap

Type: Rubber elastic bandage: Ace® Bandage or Coban® (MedicalSurgical Division/3M, St. Paul, MN)

Securing Materials

Type: Zonas® porous athletic tape (Johnson & Johnson Medical Inc., Arlington, TX)

Size: 2 in. wide

Elizabethan or Similar Collars

Optional

Animal Species

New Zealand White Rabbit

Procedure

This study is generally initiated in a stepwise approach. Any preliminary information concerning the test substance is examined including characterization, pH, *in vitro* studies, etc. This study is generally initiated with a single animal at the lowest exposure time (e.g., 3 min). If no indication of severe dermal irritation is apparent, then the study continues with a 1 h exposure and then repeated with a 4 h exposure. If no indication of severe dermal irritation is apparent, then the study may continue with the two remaining animals at the appropriate exposure period. The hair is removed from a sufficient area on the rabbit's back on the day before dosing. Care should be taken to avoid abrading the skin during the clipping procedure. On the day of dosing, each test site (approximately 1 × 1 in.² of intact skin) should be selected based on the number of exposure periods required (i.e., 3 min, 1 h, and/or 4 h). The 1 × 1 in. gauze dressing should be secured to the animal's back at the designated exposure site on at least two cut edges of the gauze patch using the nonirritating Blenderm tape. The test substance, either 0.5 mL or 0.5 g, should be administered under the gauze dressing at each exposure site and the remaining cut edges secured to the

animal's back. Liquids and powders should be administered as received. If the test article is a solid or powder that does not work well as a paste (e.g., does not spread well), 0.5 g of the test article will be applied to an approximate 1 × 1 in. 4-ply gauze patch and will be moistened with the appropriate amount of distilled water or suitable vehicle (generally 0.5 mL) and the gauze patch applied to the test site. The elastic wrap is then wrapped around the animal's torso and secured in place using the Zonas athletic tape. The Zonas is wrapped around the outermost portion of the elastic wrap at the cranial and caudal ends. If more than one exposure interval is utilized, the elastic wrap overlying the gauze dressings may be delineated using an indelible marker. This should aid in the unwrapping process. An Elizabethan or similar collar may then be applied to the animal. After the designated exposure interval (i.e., 3 min, 1 h, and/or 4 h), a window can be cut into the elastic wrap overlying the gauze patch for the appropriate test site, and the gauze patch removed. The test site should be delineated with an indelible marker and then rinsed with a suitable vehicle (e.g., distilled water). If more than one exposure interval is utilized, the cutout window of the elastic wrap should be secured to the animal with additional Blenderm tape. This will help prevent possible disruption of the remaining site(s). These steps are repeated until the last exposure interval is completed or significant/severe irritation is observed, at which time the entire elastic wrap is removed. If at any time, corrosion is apparent, the study may be terminated. Otherwise, at the appropriate grading intervals (e.g., after patch removal and at 24, 48, and 72 h after patch application), the animal should be examined and scored for signs of erythema and edema (and any other notable changes) according to the Draize dermal grading system. Grading of the test sites may be continued after the 72 h scoring interval if irritation persists (e.g., day 7, day 10, and day 14).

PHOTOIRRITATION TEST IN RABBITS

Materials

UVA Bulbs

Eight (nonoccluded procedure) or four (occluded procedure) Sylvania (Osram Sylvania, Danvers, MA) F40/350BL/ECO blacklight fluorescent or equivalent. Obtain spectral irradiance of the ultraviolet radiation source to demonstrate that it is a reasonable surrogate for solar sunlight in the ~290–400 nm waveband.

Photometer/Radiometer

IL 1350 radiometer/photometer (International Light, Holly, MI) or Personal Measurement Assistant (Model PMA2100, Solar Light Company, Inc., Philadelphia, PA) or equivalent.

Irradiation Deflector

Aluminum foil

Materials for Nonocclusive Procedure

Rabbit stocks or other restraining device

Materials for Occlusive Procedure

1. Occlusive Materials

Type: Impervious material: plastic wrap or other suitable plastic wrap

2. Binding Materials

Type: Rubber elastic bandage: Ace® bandage, or Coban® (Medical Surgical Division/3M, St. Paul, MN), or Expandover® (Kendall Brand/Covidien, Mansfield, MA)

Size: Adequate wrapping of the entire test site

3. Securing Materials

Type: Zonas® porous athletic tape (Johnson & Johnson Medical Inc., Arlington, TX)

Size: 2 in. wide

4. Tape—Nonirritating

Type: Blenderm®

Size: 1 in. wide

5. Gauze Dressing

Type: Ace-Tex Corporation nonsterilized

Thickness: 4-ply gauze dressing

Size: 1 × 1 in.²

Animal Species

New Zealand White Rabbit

Procedure

Preliminary Procedure

The photoirritation study is conducted using three or six adult New Zealand White rabbits per group. Animals of either sex should be utilized for the test and positive control (if utilized) groups. On the day before dose administration, the animals selected for study should have the fur clipped from the dorsal area of the trunk using a small animal clipper. Care should be taken to avoid abrading the skin during the clipping procedure.

Nonoccluded Procedure

On the following day (day 0), the test article (or positive control material, example, Oxsoralen lotion, 1% 8-MOP from CN Pharmaceuticals, Inc., Cosa Mesa, CA, if used) will be applied initially to one small area of the intact skin on the right side of each animal as follows: a 0.025 mL dose of the test article (or positive control material, if used) will be applied to one approximate 2.5 × 2.5 cm test site on the animals. The test sites will be delineated with a marker. The test sites will remain unoccluded. Immediately after application, the animals will be placed in stocks. Animals will remain in stocks until the completion of the UVA light exposure period and completion of the left site dose period. Approximately 2 h after test article application, excess test article may be removed using dry gauze to have adequate UVA light exposure. The test site may be re-delineated, if needed. Each animal will be wrapped with aluminum foil. An approximate 2.5 × 2.5 cm² section

will be cut in the aluminum foil to expose the test site on the right side. Treatment sites on the right side will then be exposed to a target dose of 5 or 10 J/cm². UVA light (320–400 nm) will be emitted from a bank of eight Sylvania F40/350BL/ECO blacklight fluorescent tubes. The peak emission of the light source will be 360 nm. After the completion of the UVA light exposure period, the foil will be removed. Any residual test article (from right test sites) will be removed with gauze moistened in deionized water (or appropriate solvent) followed by dry gauze. Animals will be removed from the stocks, and the test article (or positive control material) will be applied to an area of intact skin approximately 2.5 × 2.52 cm on the left side of each animal. The test sites will be delineated with a marker. The test site will remain unoccluded. The animals will be returned to the stocks until the completion of the exposure period. Approximately 2 h after test article application on the second test site (on the left side), any residual test article (from the left test site) will be removed with gauze moistened in deionized water (or appropriate solvent) followed by dry gauze. The test sites may be re-delineated, if needed. The animals will be returned to their cages.

Occluded Procedure

On the day of dose administration (day 0), the test article (or positive control material, if utilized) will be applied to two small areas of intact skin of each animal as follows: 0.5 mL or 0.5 g aliquots of the test substance should be applied to two separate areas of intact skin, one on the left side and one on the right side of each test animal. The test substance is held in contact with the skin using an approximately 1 × 1 in.² gauze patch secured to the animal with a nonirritating tape. An impervious plastic wrap is placed over the trunk of the animal and further wrapped with an elastic wrap. The elastic wrap is then secured to the animal using the athletic tape. Animals will be placed in stocks following dosing and will remain in stocks until the completion of the UVA light exposure period. Approximately 2 h after chamber application, the elastic wrap, plastic wrap, and gauze patch located on the right side of each animal will be removed. Excess test article may be removed using dry gauze to have adequate UVA light exposure. The test site may be re-delineated (if needed). The patch on the left side of the animal will remain undisturbed. Each animal will be wrapped with an aluminum foil. An approximate 2.5 × 2.5 cm² section will be cut in the aluminum foil to expose each test site on the right side. Treatment sites on the right side will then be exposed to a target dose of 5 or 10 J/cm². UVA light (320–400 nm) will be emitted from a bank of four or eight Sylvania F40/350BL/ECO blacklight fluorescent tubes. The peak emission of the light source will be 360 nm. After the completion of the UVA light exposure period, the foil and chamber (from the left side) will be removed. Any residual test article (from all sites) will be removed with gauze moistened in

deionized water (or appropriate solvent) followed by dry gauze. Animals will be returned to their cages.

PHOTOIRRITATION TEST IN GUINEA PIGS

Materials

UVA Bulbs

Eight Sylvania® (Osram Sylvania, Danvers, MA) F40/350BL/ECO blacklight fluorescent or equivalent. Obtain spectral irradiance of the ultraviolet radiation source to demonstrate that it is a reasonable surrogate for solar sunlight in the ~290–400 nm waveband.

Photometer/Radiometer

IL 1350 radiometer/photometer (International Light, Holly, MI) or Personal Measurement Assistant (Model PMA2100, Solar Light Company, Inc. Philadelphia, PA) or equivalent.

Irradiation Deflector

Aluminum foil

Materials for Nonocclusive Procedure

Type: Buehler restainer or similar device

Type: Dental dam

Materials for Occlusive Procedure

1. Occlusive materials

Type: 25 mm Hilltop® Chamber (Hilltop Research, Inc., Cincinnati, OH)

2. Binding materials

Type: Buehler restainer or similar device

Type: Dental dam

Animal Species

Hartley-derived albino guinea pig

Procedures

Preliminary Procedures

The photoirritation study is conducted using six adult Hartley-derived albino guinea pigs per group. Animals of either sex should be utilized for the test and positive control (if utilized) groups. On the day before dose administration, the animals selected for study should have the fur clipped from the dorsal area of the trunk of each animal using a small animal clipper. Care should be taken to avoid abrading the skin during the clipping procedure.

Nonoccluded Procedure

On the following day (day 0), immediately prior to application, the animals will be placed in individual restrainers. The dental dam will be pulled taut over the back and secured to the bottom of the restrainer. An approximate 2.5 × 2.5 cm window will be cut into the right side of the dental dam to ensure that the test sites remain unoccluded. The restrainers will be adjusted as necessary to minimize any discomfort to the animals. Animals will remain in restrainers until the completion of the UVA light exposure period and completion of the left site dose period.

The test article (or positive control material, example, Oxsoralen Lotion 1% 8-MOP from CN Pharmaceuticals, Cosa, CA, if used) will be applied initially to one small area of intact skin on the right side of each appropriate animal as follows: a 0.025 mL dose of the test article (or positive control material, if used) will be applied to one approximate 2.5×2.5 cm test site on the animals. The test sites will then be delineated with a marker. The test sites will remain unoccluded. Approximately 2 h after test article application, excess test article may be removed using dry gauze in order to have adequate UVA light exposure. The test sites may be re-delineated, if needed. The back of each animal will be covered with aluminum foil. An approximate 2.5×2.5 cm² section will be cut in the aluminum foil to expose each test site on the right side. Treatment sites on the right side will then be exposed to a target dose of 10 J/cm². UVA light (320–400 nm) will be emitted from a bank of eight Sylvania F40/350BL/ECO blacklight fluorescent tubes. The peak emission of the light source will be 360 nm. After the completion of the UVA light exposure period, the foil will be removed. Any residual test article (from right test sites) will be removed with the gauze moistened in deionized water (or appropriate solvent) followed by dry gauze. Animals will remain in restrainers, and an approximate 2.5×2.5 cm window will be cut in the dental dam on the left side. The test article (or positive control material, if used) will be applied to an area of intact skin on the left side of each animal. The test material will be applied to an approximate 2.5×2.5 cm test site on the left side of the animals. The test site will remain unoccluded. Approximately 2 h after test article application on the second test site (on the left side), the remaining dental dam will be removed. Any residual test article (from the left test site) will be removed with gauze moistened in deionized water (or appropriate solvent) followed by dry gauze. The test sites may be re-delineated, if needed. The animals will be returned to their cages.

Occluded Procedure

On the day of dose administration (day 0), the test article will be applied to two small areas of intact skin on the left and right side of each appropriate animal as follows: a 0.3 mL or 0.3 g (or maximum dose, powders to be moistened with the appropriate vehicle) dose of the test article (or positive control material as described previously, if used) will be applied to two 25 mm Hilltop chambers just prior to applying the chambers to the back. The Hilltop chambers will be applied to the back as quickly as possible. The test sites will be delineated with a marker. Immediately following application, the animals will be placed in individual restrainers, and the chamber will be held at the designated site using rubber dental dam. The dental dam will be pulled taut over the back and secured to the bottom of the restrainer. The restrainers will be adjusted as necessary to minimize any discomfort to the animals. Animals will remain in restrainers until the completion of the UVA light exposure period. Approximately 2 h after chamber

application, an approximate 2.5×2.5 cm² will be cut into the dental dam and the chamber located on the right side of each animal will be removed. Excess test article may be removed using dry gauze to have adequate UVA light exposure. The test site may be re-delineated (if needed). The chamber on the left side of the animal will remain undisturbed. The back of each animal will be covered with aluminum foil. An approximate 2.5×2.5 cm² section will be cut in the aluminum foil to expose each test site on the right side. Treatment sites on the right side will then be exposed to a target dose of 10 J/cm². UVA light (320–400 nm) will be emitted from a bank of eight Sylvania F40/350BL/ECO blacklight fluorescent tubes. The peak emission of the light source will be 360 nm. After the completion of the UVA light exposure period, the foil, the remaining dental dam, and the chamber (from the left side) will be removed. Any residual test article (from all sites) will be removed with the gauze moistened in deionized water (or appropriate solvent) followed by dry gauze.

MATERIALS AND PROCEDURES FOR PERFORMING DERMAL SENSITIZATION STUDIES^{5–14,38–54}

MODIFIED BUEHLER SENSITIZATION TEST IN GUINEA PIGS

Materials

Occlusive Materials

Type: 25 mm Hilltop[®] chamber (Hilltop Research, Inc., Cincinnati, OH), 2 × 2 cm Webriil[®] cotton pad with adhesive tape (Covidien, Mansfield, MA)

Binding Materials

Elastic wrap

Type: Expandover[®] (Kendall Brand/Covidien, Mansfield, MA), Coban[®] (Medical Surgical Division/3M, St. Paul, MN)

Securing Materials

Type: Conform[®] (Kendall Brand/Covidien Mansfield, MA)

Size: 1 in. wide

Depilatory Materials

Veet[®] (Reckitt Benckiser LCC, Parsippany, NJ) hair remover cream (optional)

Animal Species

Hartley albino guinea pig

Procedure

A topical range-finding irritation screen should generally be performed before initiating the sensitization study. Four graded levels (generally 25% w/v, 50% w/v, 75% w/v, and 100%) are used for this procedure. Optimally, the topical range-finding study should produce no systemic toxicity and a spectrum of dermal responses that includes grades 0, ±, 1 and 2 unless the test substance is not dermally irritating at 100%.

Based on the range-finding results, the test substance concentration used for induction should produce no systemic toxicity and a mild to moderate dermal response (grades \pm , 1 or 2) unless the test substance is not dermally irritating at 100%.

The test substance concentration used for challenge/rechallenge should produce no systemic toxicity and dermal responses generally consisting of grades 0 to \pm unless the test substance is not dermally irritating at 100%. Generally, only one concentration is tested at challenge. If the results of the challenge procedure are not conclusive, then a rechallenge may need to be performed to clarify the challenge responses. If necessary, more than one concentration may be used for challenge/rechallenge with caution to ensure that naïve skin and proper occlusion are utilized for each test site in order to provide appropriate results.

The sensitization potential of the test article will be based on the dermal responses observed on the test and control animals at challenge and rechallenge (if conducted). Generally, dermal scores of ≥ 1 in the test animals with scores of 0 to \pm noted in the controls are considered indicative of sensitization. Dermal scores of 1 in both the test and control animals are generally considered equivocal unless a higher dermal response (\geq grade 2) is noted in the test animals. For a mild to moderate sensitizer (e.g., α -hexylcinnamaldehyde [HCA]) or a moderate to strong sensitizer (e.g., 2, 4-dinitrochlorobenzene [DNCB]) in a nonadjuvant assay, a response of at least 15% or 30%, respectively, should be expected.

Topical Range-Finding Study

On the day before dose administration, four topical range-finding guinea pigs should be weighed and the hair removed from the right and left side of the animals with a small animal clipper. Care should be taken to avoid abrading the skin during clipping procedures. On the following day, up to four closed patches/chambers at four different concentrations of test substance can be applied to the clipped area of each animal (one patch/chamber for each level of test substance). For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25 mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed) should be utilized. Before chamber application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The patches/chambers should then be applied to the clipped surface as quickly as possible. The trunk of the animal should be wrapped with elastic wrap which is secured with adhesive tape (if necessary) to prevent removal of the patch/chamber and the animal returned to its cage. Approximately 6 h after patch/chamber application, the elastic wrap, tape, and patches/chambers should be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water). The test sites of the topical range-finding animals should be graded for irritation at approximately 24 and 48 h after patch/chamber application using the Buehler dermal grading system.

Induction

On the day before the first induction dose administration (day-1), all sensitization study animals should be weighed. The hair should then be removed from the left side of the test animals with a small animal clipper. Care should be taken to avoid abrading the skin during clipping procedures. On the following day (day 0), patches/chambers containing the test substance should be applied to the clipped area of 10–20 test animals. For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25 mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed) should be utilized. Before chamber application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The patch/chamber should then be applied to the clipped surface as quickly as possible. The trunk of each animal should be wrapped with elastic wrap which is secured with an adhesive tape (if necessary) to prevent removal of the patch/chamber and the animal returned to its cage. Approximately 6 h after dosing, the elastic wrap, tape, and patch/chamber will be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water). The induction clipping, patch application, and grading procedure should be repeated on study day 7 (± 1 day) and study day 14 (± 1 day) so that a total of three consecutive induction exposures should be administered to the test animals. Test sites should be graded for dermal irritation at approximately 24 and 48 h after patch application using the Buehler dermal grading system. The application site may be moved if irritation persists from a previous induction exposure but will remain on the left side of the animal. If a positive control group is necessary, DNCB or HCA is an acceptable positive control substance, and a positive control group consisting of 10 DNCB or HCA test animals and 10 DNCB or HCA control animals may be used. The DNCB or HCA test and DNCB or HCA control animals should be treated in the same manner as the sensitization study test and challenge control animals throughout the study. The DNCB concentrations standardly used for induction and challenge are 0.1%–0.5% w/v and 0.05%–0.1% w/v, respectively. The HCA concentrations standardly used for induction and challenge are 3.0%–5.0% and 1.0%–2.5%, respectively.

Challenge

On the day before challenge dose administration, the hair should be removed from the right side of the test and challenge control animals with a small animal clipper. Care will be taken to avoid abrading the skin during clipping procedures. On the next day (day 28 ± 1 day), patches/chambers containing the test substance should be applied to a naïve site within the clipped area of the test and challenge control animals. For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25 mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed)

should be used. Before chamber application, the test substance should be moistened with an appropriate vehicle (e.g., distilled water). The patch/chamber should then be applied to the clipped surface as quickly as possible. The trunk of each animal should be wrapped with elastic wrap which is secured with adhesive tape (if necessary) to prevent removal of the patch/chamber, and the animal returned to its cage. Approximately 6 h after dosing, the elastic wrap, tape, and patch/chamber should be removed. The test substance should then be removed with a suitable vehicle (e.g., distilled water).

Approximately 20 h after patch/chamber removal, the test sites may be depilated (optional) as follows:

1. Depilatory (e.g., Veet Hair Remover cream) should be placed on the test sites and surrounding areas and left on for no more than 15 min.
2. The depilatory should then be thoroughly removed with a stream of warm water. The animals should be dried with a towel and returned to their cages.

Note: The depilatory process has an advantage of being able to view test sites without hair; however, from time to time, they may produce confounding primary irritation, or suspected test article/depilatory reactions may be observed producing unanticipated dermal responses in the test and control animals. In addition, the manufacturer may change ingredients without warning. For these reasons, depilatories require qualification for laboratory use. Scoring closely shaved test sites can be done appropriately with proper training.

Test sites should be graded for dermal irritation at approximately 24 and 48 h after patch removal using the Buehler dermal grading system.

Rechallenge

If a rechallenge phase is required, the procedure should be performed on day 35 (± 1 day). The animal's haircoat should again be clipped on the right side of the animal on the day before dosing. The exposure period, dosing, wrapping, and depilation (if used) procedures should be the same as used in the challenge procedure except that the 10–20 test and 10 naïve rechallenge control animals and a naïve skin site are utilized.

STANDARD BUEHLER SENSITIZATION TEST IN GUINEA PIGS

Materials

Occlusive Materials

Type: 25 mm Hilltop® Chamber (Hilltop Research, Inc., Cincinnati, OH), 2 × 2 cm Webril® Patch cotton pad with adhesive tape (Covidien, Mansfield, MA)

Binding Materials

Elastic wrap

Type: Expandover® (Kendall Brand/Covidien, Mansfield, MA), Coban® (Medical Surgical Division/3M, St. Paul, MN)

Securing Materials

Type: Conform® (Kendall Brand/Covidien Mansfield, MA)

Size: 1 in. wide

Depilatory Materials

Veet® (Reckitt Benckiser LCC, Parsippany, NJ) hair remover cream (optional)

Animal Species

Hartley albino guinea pig

Procedure

A topical range-finding irritation screen generally should be performed before initiating the sensitization study. Four graded levels (generally 25% w/v, 50% w/v, 75% w/v, and 100%) are utilized for this procedure. Optimally, the topical range-finding study should produce no systemic toxicity and a spectrum of dermal responses that includes grades 0, \pm , 1, and 2 unless the test substance is not dermally irritating at 100%.

Based on the range-finding results, the test substance concentration used for induction should produce no systemic toxicity and a mild-to-moderate dermal response (grades \pm , 1 or 2), unless the test substance is not dermally irritating at 100%. The test substance concentration may be varied (increased or decreased) during the induction period depending on the dermal responses produced.

The test substance concentration used for challenge/rechallenge should produce no systemic toxicity and dermal responses generally consisting of grades 0 to \pm unless the test substance is not dermally irritating at 100%. Generally, only one concentration is tested at challenge. If the results of the challenge procedure are not conclusive, then a rechallenge may need to be performed to clarify the challenge responses. If necessary, more than one concentration may be used for challenge/rechallenge with caution to ensure that naïve skin and proper occlusion are utilized for each test site in order to provide appropriate results.

The sensitization potential of the test article will be based on the dermal responses observed on the test and control animals at challenge and rechallenge (if conducted). Generally, dermal scores of ≥ 1 in the test animals with scores of 0 to \pm noted in the controls are considered indicative of sensitization. Dermal scores of 1 in both the test and control animals are generally considered equivocal unless a higher dermal response (\geq grade 2) is noted in the test animals. For a mild-to-moderate sensitizer (e.g., α -HCA) or a moderate-to-strong sensitizer (e.g., 2, 4-DNCB), in a non-adjuvant assay, a response of at least 15% or 30%, respectively, should be expected.

Topical Range-Finding Study

On the day before dose administration, four topical range-finding guinea pigs should be weighed and the hair removed from the right and left sides of the animals with a small animal clipper. Care should be taken to avoid abrading the skin during clipping procedures. On the next day, up to four closed patches/chambers at four different concentrations of

test substance can be applied to the clipped area of each animal (one patch/chamber for each level of test substance). For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL will be placed on a 25-mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed) should be utilized. Before chamber application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The patches/chambers should then be applied to the clipped surface as quickly as possible. The trunk of the animal should be wrapped with an elastic wrap which is secured with adhesive tape (if necessary) to prevent removal of the patch/chamber, and the animal returned to its cage. Approximately 6 h after patch/chamber application, the elastic wrap, tape, and patches/chambers should be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water). The test sites of the topical range-finding animals should be graded for irritation at approximately 24 and 48 h after patch/chamber application using the Buehler dermal grading system.

Induction

On the day before the first induction dose administration (day-1), all sensitization study animals should be weighed. The hair should then be removed from the left side of the test animals with a small animal clipper using a No. 40 clipper blade. Care should be taken to avoid abrading the skin during clipping procedures. On the next day (day 0), patches/chambers containing the test substance should be applied to the clipped area of 10–20 test animals. For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25-mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25-mm Hilltop Chamber (with cotton pad removed) should be used. Before chamber application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The patch/chamber should then be applied to the clipped surface as quickly as possible. The trunk of each animal should be wrapped with an elastic wrap which is secured with adhesive tape (if necessary) to prevent removal of the patch/chamber and the animal returned to its cage. Approximately 6 h after dosing, the elastic wrap, tape, and patch/chamber will be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water). The induction clipping, patch application, and grading procedure should be repeated three times a week (i.e., Monday, Wednesday, Friday) for three consecutive weeks so that a total of nine consecutive induction exposures are administered to the test animals. Test sites should be graded for dermal irritation at approximately 24 and 48 h after patch application using the Buehler dermal grading system. The application site may be moved if irritation persists from a previous induction exposure but will remain on the left side of the animal. If a positive control group is necessary, DNCB or HCA is an acceptable positive control substance, and a positive control group consisting of 10 DNCB or HCA

test animals and 10 DNCB or HCA control animals may be used. The DNCB or HCA test and DNCB or HCA control animals should be treated in the same manner as the sensitization study test and challenge control animals throughout the study. The DNCB concentrations standardly used for induction and challenge are 0.1%–0.5% w/v and 0.05%–0.1% w/v, respectively. The HCA concentrations standardly used for induction and challenge are 3.0%–5.0% and 1.0%–2.5%, respectively.

Challenge

On the day before challenge dose administration, the hair should be removed from the right side of the test and challenge control animals with a small animal clipper. Care should be taken to avoid abrading of the skin during clipping procedures. On the next day (day 32 \pm 1 day), patches/chambers containing the test substance should be applied to a naive site within the clipped area of the test and challenge control animals. For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25 mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed) should be utilized. Before chamber application, the test substance should be moistened with an appropriate vehicle (e.g., distilled water). The patch/chamber should then be applied to the clipped surface as quickly as possible. The trunk of each animal should be wrapped with an elastic wrap which is secured with an adhesive tape (if necessary) to prevent removal of the patch/chamber and the animal returned to its cage. Approximately 6 h after dosing, the elastic wrap, tape, and patch/chamber should be removed. The test substance should then be removed with a suitable vehicle (e.g., distilled water).

Approximately 20 h after patch/chamber removal, the test sites may be depilated (optional) with a qualified material as follows:

1. Depilatory (e.g., Veet Hair Remover cream) should be placed on the test sites and surrounding areas and left on for no more than 15 min.
2. The depilatory should then be removed thoroughly with a stream of warm water. The animals should then be dried with a towel and returned to their cages.

Note: The depilatory process has an advantage of being able to view test sites without hair; however, from time to time, they may produce confounding primary irritation, or suspected test article/depilatory reactions may be observed producing unanticipated dermal responses in the test and control animals. In addition, the manufacturer may change ingredients without warning. For these reasons, depilatories require qualification for laboratory use. Scoring closely shaved test sites can be done appropriately with proper training.

Test sites should be graded for dermal irritation at approximately 24 and 48 h after patch removal using the Buehler dermal grading system.

Rechallenge

If the results of the challenge procedure are not conclusive, then a rechallenge may be needed to clarify the challenge responses.

If a rechallenge phase is required, the procedure should be performed on day 39 (± 1 day). The animal's haircoat should again be clipped on the right side of the animal on the day before dosing. The exposure period, dosing, wrapping, and depilation procedures should be the same as used in the challenge procedure except that the 10–20 test and 10 naïve rechallenge control animals and a naïve skin site are utilized.

GUINEA PIG MAXIMIZATION TEST

Materials

Injection Materials

1-cc disposable syringe, 25–27 5/8 gauge needle

Occlusive Materials

Type: 2 × 2 cm Webril® cotton pad with adhesive tape, 2 × 4 cm Modified Webril® cotton pad with adhesive tape (Covidien, Mansfield, MA), 25 mm Hilltop® Chamber (Hilltop Research, Inc., Cincinnati, OH)

Binding Materials

Elastic wrap

Type: Coban® (Medical Surgical Division/3M, St. Paul, MN)

Securing Materials

Type: Conform® (Kendall Brand/Covidien Mansfield, MA), or Zones® Athletic Tape (Johnson & Johnson Medical Inc., Arlington, TX)

Size: 1-in. wide

Animal Species

Hartley albino guinea pig

Procedure

For the topical screen, four graded levels (generally 25% w/v, 50% w/v, 75% w/v, and 100%) are used for this procedure. Optimally, the topical range-finding study should produce no systemic toxicity and a spectrum of dermal responses that includes grades 0, \pm , 1 and 2 unless the test substance is not dermally irritating at 100%.

For the intradermal screen, four graded levels (generally 0.1% w/v, 1.0% w/v, 3.0% w/v, and 5.0% w/v) are used for this procedure. Optimally, the intradermal range-finding study should produce no systemic toxicity, but only localized reactions at the injection site (responses that do not notably extend beyond the site of injection).

Based on this information, the test substance concentration used for intradermal induction should produce no systemic toxicity, but only localized reactions at the injection site (responses that do not notably extend beyond the site of injection). For topical induction, the test substance concentration used should produce a mild-to-moderate dermal response (grades ± 1 or 2) unless the test substance is not dermally irritating at 100%.

The test substance concentration used for challenge/rechallenge should produce no systemic toxicity and dermal responses generally consisting of grades 0 to \pm unless the test substance is not dermally irritating at 100%. Generally, only one concentration is tested at challenge. If the results of the challenge procedure are not conclusive, then a rechallenge may need to be performed to clarify challenge responses. If necessary, more than one concentration may be used for challenge/rechallenge with caution to ensure that naïve skin and proper occlusion are utilized for each test site in order to provide appropriate results.

The sensitization potential of the test article will be based on the dermal responses observed on the test and control animals at challenge and rechallenge (if conducted). Generally, dermal scores of ≥ 1 in the test animals with scores of 0 to \pm noted in the controls are considered indicative of sensitization. Dermal scores of 1 in both the test and control animals are generally considered equivocal unless a higher dermal response (\geq grade 2) is noted in the test animals. For a mild-to-moderate sensitizer (e.g., α -HCA) in an adjuvant assay, a response of at least 30% should be expected.

Topical Range-Finding Study

On the day before dose administration, four topical range-finding guinea pigs should be weighed and the hair removed from the right and left sides of the animals with a small animal clipper. Care should be taken to avoid abrading the skin during clipping procedures. On the next day, up to four closed patches/chambers at four different concentrations of test substance can be applied to the clipped area of each animal (one patch/chamber for each level of the test substance). For liquids, gels and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25 mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed) should be used. Before chamber application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The patches/chambers should then be applied to the clipped surface as quickly as possible. The trunk of the animal should be wrapped with an elastic wrap which is secured with an adhesive tape (if necessary) to prevent removal of the patch/chamber. Approximately 24 h after patch/chamber application, the elastic wrap, tape, and patches/chambers should be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water). The test sites of the topical range-finding animals should be graded for irritation at approximately 24 and 48 h after patch/chamber removal using the Buehler dermal grading system.

Intradermal Range-Finding Study

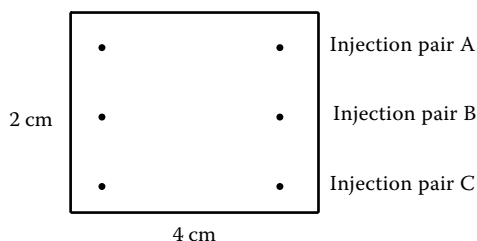
On the day before dose administration, four intradermal range-finding guinea pigs should be weighed and the hair removed from the right and left sides of the animals with a small animal clipper. Care should be taken to avoid abrading the skin during clipping procedures. On the next day, up to

four intradermal injections at four different concentrations of test substance can be injected into the clipped area of each animal (one injection for each level of the test substance). A dose of 0.1 mL should be injected for each concentration using a syringe attached to a hypodermic needle.

The test sites of the intradermal range-finding animals should be graded for irritation at approximately 24 and 48 h after intradermal injections using the Buehler dermal grading system.

Induction

On the day before intradermal dosing (day-1), all sensitization study animals should be weighed. The hair should then be removed from the scapular area of 10–20 test and 10 challenge control animals with a small animal clipper. Care should be taken to avoid abrading the skin during clipping procedures. On the next day (day 0), three pairs of intradermal injections should be made in the clipped area of all sensitization study animals. The injections should be kept within an approximate 2 × 4 cm area with one row of three injections on each side of the backbone as indicated in the following:



Injections for the test animals should be as indicated: (1) Injection pair A, 0.1 mL of a 1:1 v/v Freund's Complete Adjuvant in sterile water emulsion (FCA emulsion); (2) Injection pair B, 0.1 mL of the test substance preparation; (3) Injection pair C, 0.1 mL of the test substance in the FCA emulsion.

Injections for the challenge control animals should be as indicated: (1) Injection pair A, 0.1 mL of the FCA emulsion; (2) Injection pair B, 0.1 mL of the vehicle; (3) Injection pair C, 0.1 mL of the vehicle/ FCA emulsion.

On the day before topical induction, the hair should be clipped from the scapular area of the test and challenge control animals using a small animal clipper. Care should be taken to avoid abrading the skin during the clipping procedures. If the test substance is nonirritating, a 10% w/w sodium lauryl sulfate (SLS) preparation in petrolatum may be applied to the 2 × 4 cm intradermal injection area so that the injection area is sufficiently covered with the preparation (0.5 mL). On the next day (day 7 ± 1 day), any residual SLS preparation should be removed with a dry gauze, and the test animals should receive a topical dose of the test substance. The challenge control animals should receive a topical dose of the vehicle. Each animal's dose first should be applied to a modified Webril patch, and the patch applied over the intradermal injection sites as quickly as possible. For liquids, gels, and pastes, a dose of 0.8 mL should be

placed on the modified Webril patch. For solids and powders, the maximum volume of solid/powder that can be maintained on the modified Webril patch should be used. Before solid/powder application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The trunk of each animal should then be wrapped with an elastic wrap which is secured with adhesive tape (if necessary) to prevent removal of the patch and the animal returned to its cage. Approximately 48 h after dosing, the elastic wrap, tape, and patch should be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water).

If a positive control group is necessary, DNCB or HCA are acceptable positive control substances, and a positive control group consisting of 10 DNCB or HCA test animals and 10 DNCB or HCA control animals may be used. The DNCB or HCA test and control animals should be treated in the same manner as the sensitization study test and control animals throughout the study. The DNCB concentrations standardly used for induction and challenge are 0.1%–0.5% w/v and 0.05%–0.1% w/v, respectively. The HCA concentrations standardly used for induction and challenge are 3.0%–5.0% w/v and 0.5%–1.0% w/v, respectively.

Challenge

On the day before challenge dose administration, all test and challenge control animals should be weighed. The hair should then be removed from the right side of the test and challenge control animals with a small animal clipper. Care should be taken to avoid abrading of the skin during clipping procedures. On the next day (day 21 ± 1 day), the test and challenge control animals should receive a topical dose of the test substance. For liquids, gels, and pastes, a dose of 0.3 or 0.4 mL should be placed on a 25 mm Hilltop Chamber or Webril patch. For solids and powders, the maximum volume of solid/powder that can be contained in a 25 mm Hilltop Chamber (with cotton pad removed) should be used. The weight of the solid/powder placed in the chamber should be recorded. Before chamber application, the test substance should be moistened with a suitable vehicle (e.g., distilled water). The patch/chamber should then be applied to the clipped surface as quickly as possible. The trunk of each animal should be wrapped with an elastic wrap which is secured with an adhesive tape (if necessary) to prevent removal of the patch/chamber and the animal returned to its cage. Approximately 24 h after dosing, the elastic wrap, tape, and patch/chamber should be removed. The test substance should be removed with a suitable vehicle (e.g., distilled water). The test sites should be graded for dermal irritation at approximately 24 and 48 h after patch removal using the Buehler dermal grading system.

Rechallenge

If a rechallenge phase is required, the rechallenge procedure should be performed on day 28 (±1 day). The animal's haircoat should be clipped on the left side of the animal on the day before dosing. The exposure period, dosing, and the

wrapping procedures are the same as used in the challenge procedure except that 10 naive rechallenge control animals and a naive skin site are utilized.

MURINE LOCAL LYMPH NODE ASSAY (RADIOLABELED DESIGN)

Materials

Dosing Materials

1. Calibrated pipette or syringe
2. 1-cc disposable syringe, 25–27 gauge needle

Lymph Node Collection/Cell Suspension Materials

1. Tissue culture dish (e.g., 60 mm)
2. Tissue culture tube (e.g., 12 × 75 mm)
3. Centrifuge tube (e.g., 15 mL)
4. Nylon or stainless steel screen, ~100–200 µm mesh opening, ~85 µm thick
5. Pasteur pipette
6. Forceps
7. Scintillation cocktail (e.g., Ecovolume®)

Animal Species

Female CBA/Ca or CBA/J mice.

Procedure

The test material is applied directly to the ears for assessing the contact hypersensitization. These procedures evaluate the ability of the test article to cause lymphocyte proliferation as determined by incorporation of 3H-thymidine by lymphocytes within the appropriate draining lymph nodes of topically treated mice, which is then compared to appropriate control mice. Generally, no prescreen or range-finding animals are utilized unless there is a concern for dermal trauma (corrosion/severe irritation) or systemic toxicity. Instead, at least three consecutive concentrations from the following range are utilized: 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1% (w/v). The selection is made to provide the highest possible test concentration, which is generally limited by compatibility with the vehicle chosen and the suitability of the resulting preparation for unoccluded dermal application. Vehicle should not bias the test result. The following vehicles are recommended, in order of preference: acetone–olive oil (4:1), acetone, N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethylsulfoxide or other appropriate vehicles. In some cases, the clinically solvent or commercial formulation may be needed as an additional control. Aqueous vehicles are not normally recommended because of insufficient absorption during the dosing procedure; however, aqueous–organic mixtures such as 3:1 acetone–water or 80% ethanol have been used successfully. Materials for positive control include 25% α-HCA in acetone:olive oil (4:1, v/v), 5% in 2-mercaptobenzothiazole dimethylformamide, 2,4-DNCB, and benzocaine. The ratio of the mean proliferation in each test or positive control group to the concurrent vehicle group is termed the SI; further, the SI should be ≥3.0 to be demonstrative of sensitization.

However, the strength of the dose–response relationship, the statistical significance, and the consistency of the solvent/vehicle and positive control responses may be used to determine whether the result is borderline.

Prescreen Test (If Conducted)

The prescreen test may be used to provide guidance for dose levels when systemic toxicity and/or excessive local skin irritation data are not available. The test will be conducted similar to the main mouse local lymph node assay (LLNA) study but with fewer animals (1–2) per group and no lymph node assessment. Animals will be observed for clinical signs including dermal assessment of the ears using a dermal grading system and changes in body weight. In addition, ear thickness measurements may be collected (e.g., digital micrometer) on day 0 (predose), day 2 (approximately 48 h after the first dose), and day 5 (termination). Excessive local irritation is indicated by irritation ≥ moderate to severe erythema and/or an increase in ear thickness of ≥25% on any measurement day. The highest dose selected for the main LLNA study will be the next lower dose in the prescreen that does not cause systemic toxicity and/or excessive local skin irritation.

Topical Induction

On day 0, five females per test article concentration and control group (and positive control test and control group, if utilized) will be weighed, and 25 µL of test article will be applied to the dorsal surfaces of the left and right ears. Care will be taken to ensure that the test article will not run off from the ear during application. Approximately 24 h later (day 1) and 48 h later (day 2), each animal will receive additional applications as described previously. The animals will then be rested for 2 days. The vehicle control animals (and positive control animals, if used) are treated the same as earlier.

Injection of 3H-Thymidine for Lymphocyte Incorporation

On day 5 (approximately 72 h after the final application), the five females per group will be weighed and receive an intravenous injection of 3H-thymidine for lymphocyte incorporation. The injection will consist of 0.250 mL of phosphate-buffered saline (PBS) containing 20 µCi of 3H-thymidine (specific activity of 5.0 or 6.7 Ci/mmol). An animal will be excluded from the study if the full 0.250 mL of 3H-thymidine/PBS is not properly injected intravenously.

Lymph Node Collection

Approximately 5 h after the 3H-thymidine injections, the animals should be euthanized with the appropriate humane method. The appropriate draining (auricular) lymph nodes should be removed and pooled for each individual animal. Care should be taken to assure that the lymph nodes are removed intact and placed in a capped tissue culture tube (e.g., 12 × 75 mm) containing 4 mL of PBS.

Cell Suspension

The lymph nodes will be transferred to a tissue culture dish (e.g., 60 mm) by pouring the PBS tube containing the lymph

nodes. The lymph nodes will be mechanically passed through a nylon or stainless steel screen. A pasteur pipette and a small pair of forceps will be used to rinse the screen with PBS into the tissue culture dish, which will then be rinsed with the PBS back into the culture tube to allow the capsule debris to settle to the bottom. The PBS will then be carefully drawn off with a pasteur pipette and will be placed in a centrifuge tube containing 6 mL of PBS (approximately 10 mL total tube volume). The cell suspensions will then be centrifuged and then resuspended in 20 mL of PBS, and a second wash will be performed. After completion of the second wash, the cells will be suspended in 3 mL of 5% trichloroacetic acid (w/v TCA in deionized water) and left at approximately 4°C for approximately 18 h.

Scintillation Counting

The cell suspensions will be centrifuged and resuspended in 1 mL of 5% TCA. The individual cell suspensions will be transferred into the appropriate scintillation vials containing 10 mL of scintillation cocktail along with an additional 1 mL of TCA, which has been used to rinse the bottom portion of the tube. The TCA and scintillation fluid will be thoroughly mixed by gently swirling the contents of the vial until the solution becomes clear. The sample will then be counted and recorded in disintegrations per minute (DPM).

MURINE LOCAL LYMPH NODE ASSAY

(DA TEST METHOD, A NONRADIOACTIVE ASSAY)

Materials

Dosing Materials

1. Calibrated pipette or syringe
2. 1-cc disposable syringe, 25–27 gauge needle

Lymph Node Collection/Cell Suspension Materials

1. Tissue culture dish (e.g., 60 mm)
2. Tissue culture tube (e.g., 12 × 75 mm)
3. Centrifuge tube (e.g., 15 mL)
4. Nylon or stainless steel screen, ~100–200 µm mesh opening, ~85 µm thick
5. Pasteur pipette
6. Forceps
7. ATP commercial measurement kit

Animal Species

Female CBA/J mice.

Procedure

The test material is applied directly to the ears for assessing the contact hypersensitization. These procedures evaluate the ability of the test article to cause lymphocyte proliferation as determined by quantifying adenosine triphosphate (ATP) content via bioluminescence (known to correlate with live cell number) as an indicator of lymphocyte proliferation within the appropriate draining lymph nodes of topically treated mice, which is then compared to appropriate control mice. Generally, no prescreen or range-finding animals are utilized unless there is a concern for dermal trauma (corrosion/severe irritation) or systemic toxicity. Instead, at least three

consecutive concentrations from the following range are utilized: 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1% (w/v). The selection is made to provide the highest possible test concentration, which is generally limited by compatibility with the vehicle chosen and the suitability of the resulting preparation for unoccluded dermal application. Vehicle should not bias the test result. The following vehicles are recommended, in order of preference: acetone–olive oil (4:1), acetone, N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethylsulfoxide or other appropriate vehicles. In some cases, the clinical solvent or commercial formulation may be needed as an additional control. Aqueous vehicles are not normally recommended because of insufficient absorption during the dosing procedure; however, aqueous–organic mixtures such as 3:1 acetone–water or 80% ethanol have been used successfully. Materials for positive control include 25% α -HCA in acetone:olive oil (4:1, v/v), and 25% eugenol in acetone:olive oil (4:1, v/v). The ratio of the mean proliferation in each test or positive control group to the concurrent vehicle group is termed the SI; further, the SI should be ≥ 1.8 to be demonstrative of sensitization. However, the strength of the dose–response relationship, the statistical significance, and the consistency of the solvent/vehicle and positive control responses may be used to determine whether the result is borderline (i.e., SI value between 1.8 and 2.5 for this assay).

Prescreen Test (If Conducted)

The prescreen test may be used to provide guidance for dose levels when systemic toxicity and/or excessive local skin irritation data are not available. The test will be conducted similar to the main LLNA study but with fewer animals (1–2) per group and no lymph node assessment. Animals will be observed for clinical signs including dermal assessment of the ears using a dermal grading system and changes in body weight. In addition, ear thickness measurements may be collected (e.g., digital micrometer) on day 0 (predose), day 2 (approximately 48 h after the first dose), and day 5 (termination). Excessive local irritation is indicated by irritation \geq moderate-to-severe erythema and/or an increase in ear thickness of $\geq 25\%$ on any measurement day. In addition, a statistical increase in treated ear thickness compared to control mice may indicate an irritant. The highest dose selected for the main LLNA study will be the next lower dose in the prescreen that does not cause systemic toxicity and/or excessive local skin irritation.

Topical Induction

On day 0, five (minimum of four) females per test article concentration and control group (and positive control test and control group, if utilized) will be weighed and pretreated with 1% SLS aqueous solution to the entire dorsum of each ear (e.g., applied with four to five brush strokes per ear). After 1 h, 25 µL of test article will be applied to the dorsal surfaces of the left and right ears. Care will be taken to ensure that the test article will not run off from the ear during application. Approximately 24 h later (day 1) and 48 h later (day 2),

each animal will receive additional 1% SLS pretreatments and test substance applications as described previously. The animals will be rested for 3 days (days 3, 4, and 5) but will receive an additional 1% SLS pretreatment and test substance application on day 6. The vehicle control animals (and positive control animals, if used) are treated the same as earlier.

Lymph Node Collection

On day 7 (approximately 24–30 h postdose), the five females per group will be weighed and humanely euthanized. The appropriate draining (auricular) lymph nodes will be removed and pooled for each individual animal. Care will be taken to assure that the lymph nodes are removed intact and placed in a capped tissue culture tube (e.g., 12 × 75 mm) containing PBS. Note: Further evaluations such as dermal grading or ear thickness measurements may also be collected on day 7 prior to termination.

Cell Suspension

A single cell suspension of lymph nodes will be excised bilaterally by sandwiching the lymph nodes between two glass slides and applying pressure to crush and thinly spread the lymph nodes. The lymph node tissue from both slides will be rinsed with a total volume of 1 mL of PBS and scraped with a cell scraper into a tissue culture dish (e.g., 60 mm). The resulting lymph node cell suspension should be homogenized lightly with a cell scraper. A 20 μ L aliquot of the suspension is then collected with a micropipette, taking care not to take up the membrane that is visible to the eye, and subsequently mixed with 1.98 mL of PBS to yield a 2 mL sample. A second 2 mL sample is then prepared using the same procedure so that two samples are prepared for each animal.

Determination of Cellular Proliferation (Measuring ATP Content in Lymphocytes)

Increases in ATP content in the lymph nodes are measured by luciferin/luciferase method using an ATP measurement kit, which measures bioluminescence in Relative Luminescence Units (RLU). The assay time from the time of animal sacrifice to measurement of ATP content for each individual animal should be kept uniform, targeting approximately 30 min, because the ATP content is considered to gradually decrease with time. Thus, the series of procedures from excision of the auricular lymph nodes to ATP measurement should be completed within 20 min by the predetermined time schedule which is the same for each animal. ATP luminescence should be measured in each 2 mL sample so that a total of two ATP measurements are collected for each animal.

MURINE LOCAL LYMPH NODE ASSAY (BrdU-ELISA OR ENZYME-LINKED IMMUNOSORBENT ASSAY, A NONRADIOACTIVE ASSAY)

Materials

Dosing Materials

1. Calibrated pipette or syringe
2. 1-cc disposable syringe, 25–27 gauge needle

Lymph Node Collection/Cell Suspension Materials

1. Tissue culture dish (e.g., 60 mm)
2. Tissue culture tube (e.g., 12 × 75 mm)
3. Centrifuge tube (e.g., 15 mL)
4. Nylon or stainless steel screen, ~100–200 μ m mesh opening, ~85 μ m thick
5. Pasteur pipette
6. Forceps
7. Cell proliferation–BrdU ELISA commercial kit (Roche Applied Science, Mannheim, Germany)

Animal Species

Female CBA/JN mice.

Procedure

The test material is applied directly to the ears for assessing the contact hypersensitization. These procedures evaluate the ability of the test article to cause lymphocyte proliferation as determined by incorporation of BrdU (analog of thymidine which is similarly incorporated) by lymphocytes within the appropriate draining lymph nodes of topically treated mice, which is then compared to appropriate control mice. Generally, no prescreen or range-finding animals are utilized unless there is a concern for dermal trauma (corrosion/severe irritation) or systemic toxicity. Instead, at least three consecutive concentrations from the following range are utilized: 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1% (w/v). The selection is made to provide the highest possible test concentration, which is generally limited by compatibility with the vehicle chosen and the suitability of the resulting preparation for unoccluded dermal application. Vehicle should not bias the test result. The following vehicles are recommended, in order of preference: acetone–olive oil (4:1), acetone, N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethylsulfoxide or other appropriate vehicles. In some cases, the clinically solvent or commercial formulation may be needed as an additional control. Aqueous vehicles are not normally recommended because of insufficient absorption during the dosing procedure; however, aqueous–organic mixtures such as 3:1 acetone–water or 80% ethanol have been used successfully. Materials for positive control include 25% α -HCA in acetone:olive oil (4:1, v/v) and 25% eugenol in acetone:olive oil (4:1, v/v). The ratio of the mean proliferation in each positive control group to the concurrent vehicle group is termed the SI; further, the SI should be ≥ 1.6 to be demonstrative of sensitization. However, the strength of the dose–response relationship, the statistical significance, and the consistency of the solvent/vehicle and positive control responses may be used to determine whether the result is borderline (i.e., SI value between 1.6 and 1.9 for this assay).

Prescreen Test (If Conducted)

The prescreen test may be used to provide guidance for dose levels when systemic toxicity and/or excessive local

skin irritation data are not available. The test will be conducted similar to the main LLNA study but with fewer animals (1–2) per group and no lymph node assessment. Animals will be observed for clinical signs including dermal assessment of the ears using a dermal grading system and changes in body weight. In addition, ear thickness measurements may be collected (e.g., digital micrometer) on day 0 (predose), day 2 (approximately 48 h after the first dose), and day 5 (termination). Excessive local irritation is indicated by irritation \geq moderate-to-severe erythema and/or an increase in ear thickness of $\geq 25\%$ on any measurement day. In addition, a statistical increase in treated ear thickness compared to control mice may indicate an irritant. The highest dose selected for the main LLNA study will be the next lower dose in the prescreen that does not cause systemic toxicity and/or excessive local skin irritation.

Topical Induction

On day 0, five (minimum of four) females per test article concentration and control group (and positive control test and control group, if utilized) will be weighed and 25 μ L of test article will be applied to the dorsal surfaces of the left and right ears. Care will be taken to ensure that the test article will not run off from the ear during application. Approximately 24 h later (day 1) and 48 h later (day 2), each animal will receive additional applications as described previously. The animals will then be rested for 1 day (day 3). The vehicle control animals (and positive control animals, if used) are treated the same as earlier.

Injection of BrdU (10 mg/mL) for Lymphocyte Incorporation

On day 4 (approximately 72 h after the final application), the five females per group will receive a 0.5 mL intraperitoneal injection of BrdU for lymphocyte incorporation.

Lymph Node Collection

On day 5 (approximately 24 h) after the BrdU injections, the animals will be weighed and euthanized with the appropriate humane method. The appropriate draining (auricular) lymph nodes will be removed and pooled for each individual animal. Care will be taken to assure that the lymph nodes are removed intact and placed in a capped tissue culture tube (e.g., 12 \times 75 mm) containing 4 mL of PBS. Note: Further evaluations such as dermal grading or ear thickness measurements may also be collected on day 5 prior to termination.

Cell Suspension

The lymph nodes will be transferred to a tissue culture dish (e.g., 60 mm) by pouring the PBS tube containing the lymph nodes. The lymph nodes will be mechanically passed through a nylon or stainless steel screen or other method for generating a single-cell suspension. In each case, the target volume of the local lymph node cell suspension should be adjusted to determine the optimized volume (approximately 15 mL). The optimized volume is based on

achieving a mean absorbance of within 0.1–0.2 absorbance for the negative group.

Determination of Cellular Proliferation (Measuring BrdU Content in DNA of Lymphocytes)

BrdU is measured by ELISA using a commercial kit following instructions. Basically, 100 μ L of lymph node cell suspension is added to the wells of a microplate in appropriate replicates. After fixation and denaturation of the lymph node cells, anti-BrdU antibody is added to each microplate well and allowed to react. Subsequently, the anti-BrdU antibody is removed by washing, and the substrate solution is added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is then measured. Assay test conditions must be optimized for proper results.

PHOTOSENSITIZATION TEST IN MICE

Materials

UVA Bulbs

Four Sylvania® (Osram Sylvania, Danvers, MA) F40/350BL/ECO blacklight fluorescent or equivalent. Obtain spectral irradiance of the ultraviolet radiation source to demonstrate that it is a reasonable surrogate for solar sunlight in the ~290–400 nm waveband.

UVB Bulbs

Philips® (Philips Lighting Co., Somerset, NJ) TL40W/01 RS UVB fluorescent sunlamps or equivalent. Obtain spectral irradiance of the ultraviolet radiation source to demonstrate that it is a reasonable surrogate for solar sunlight in the ~290–315 nm waveband.

Photometer/Radiometer

IL 1350 radiometer/photometer (International Light, Holly, MI) or Personal Measurement Assistant (Model PMA2100, Solar Light Company, Inc., Philadelphia, PA).

Micrometer

Dyer® (Dyer Company, Lancaster, PA) Micrometer Model D1000 or equivalent.

Irradiation Deflector

3 mm thick sheet of plate glass or equivalent (large enough to cover UVA-exposed animals to prevent UVB exposure during UVA exposure).

Micropipettor

Eppendorf® (Brinkman Instruments, Inc., Westbury, NY) 1–50 μ L micropipettor.

Animal Species

BALB/c mice

Procedure

A preliminary irritation screen should generally be included in this test to observe the degree of primary irritation the test substance may produce. Twenty female mice, separated into four dose groups (five mice/ group), are used for this

procedure. Up to four different concentrations of the test substance can be utilized. Each mouse should be placed in a restrainer, and two measurements of ear thickness should be performed using an engineer's micrometer. Two different concentrations may be utilized on each mouse, one concentration for each ear. Eight microliters of the test substance should be used for animals in groups 3 and 4, and 8 μL of the vehicle for groups 1 and 2. The appropriate material should be applied to both sides of the ear. After a 60 min waiting period, the ears should be wiped with gauze moistened with an appropriate vehicle (e.g., distilled water). Mice from groups 2 and 4 should then be exposed to 10 J/cm^2 UVA and 25–60 mJ/cm^2 UVB. Mice in groups 1 and 3 are not irradiated. At approximately 3, 24, and 48 h postirradiation, the mice should be placed back into the restraining device and ear measurements should once again be performed and recorded.

Induction

An induction phase of the photosensitization study should be initiated with an intraperitoneal injection of cyclophosphamide (CP). A dose of 200 mg/kg CP in sterile PBS at a dose volume of 10 mL/kg should be injected approximately 3 days before the first induction. Because the CP injections may induce toxicity and/or mortality, additional mice should be used for this procedure to allow for a sufficient number of animals on study. On day 0, the backs of all mice should be clipped using a small animal clipper and an appropriately sized clipper blade. Standard study designs are set up with four groups of five female mice each. The first two groups are designated as test substance groups with the remaining two groups set up as vehicle control and ultraviolet control groups, respectively. The mice designated for groups 1, 2, and 4 are induced on days 0, 1, and 2. Group 3 animals are not treated during the induction phase. Mice in groups 1 and 2 should receive 50 μL of the appropriate test substance gently rubbed into the skin on the dorsal back of each animal on each of the induction days. Mice in group 4 should receive 50 μL of the designated vehicle in the same manner. Each mouse is then placed in an individual compartment of an irradiation box with a wire lid restraining device. Approximately 60 min after application of the appropriate material, the treated area of each animal should be gently wiped against the grain of hair growth with a gauze patch moistened with an appropriate vehicle. After the wiping procedure, animals from group 2 should be returned to their respective cages and should not receive irradiation. Mice in groups 1 and 4 should be exposed to 10 J/cm^2 UVA and 25 mJ/cm^2 UVB from a distance of approximately 20 ± 1.0 cm. For the UVA exposure, a 3 mm thick sheet of plate glass should be placed over the UVA radiometer detector during irradiation measurements to filter out any UVB wavelengths that may be emitted. The mice from groups 1 and 4 are then exposed to UVA light (320–400 nm) emitted from a

bank of four Sylvania F40/350 BL blacklight fluorescent tubes. The bank of lights is positioned approximately 20 ± 1.0 cm above the irradiation boxes containing the mice for a target dose of 10 J/cm^2 . A peak emission of the UVA lights should be at 360 nm. For the UVB exposure, the mice should be positioned under a bank of eight Philips UVB fluorescent sunlamps for an exposure of 25 mJ/cm^2 UVB light. It is preferable to rotate the animals' positions for each induction exposure so that no one group is irradiated in the same location.

Challenge

A challenge phase of the photosensitization study should be performed 7 days after the first induction phase. Before challenge, each mouse should have the ear thickness measured on both ears using an engineer's micrometer (Model D-1000). Measurements should be read and recorded as $\text{mm} \times 10^{-2}$. These measurements should take place while the animal is in a restraining device. While the animal is still restrained, 8 μL of the test substance will be administered to each side of one ear. The vehicle is then applied to both sides of the opposite ear. After approximately 60 min, the ears should be wiped with the appropriate vehicle and the animals in groups 1, 3, and 4 exposed to 10 J/cm^2 UVA and 25 mJ/cm^2 UVB as indicated previously. The group 2 mice should not be irradiated. Ear thickness measurements as described previously should be performed at approximately 24 and 48 h after the challenge procedure.

Rechallenge

If a rechallenge phase is required, the procedure should be performed 7 days after the challenge exposure. The exposure period, irradiation, and ear measurement procedures should be the same as used in the challenge procedure.

PHOTOSENSITIZATION TEST IN GUINEA PIGS

Materials

UVA Bulbs

Four Sylvania® (Osram Sylvania, Danvers, MA) F40/350/BL/ECOBAL blacklight fluorescent or equivalent. Obtain spectral irradiance of the ultraviolet radiation source to demonstrate that it is a reasonable surrogate for solar sunlight in the ~290–400 nm waveband.

Photometer/Radiometer

IL 1350 radiometer/photometer (International Light, Holly, MI) or Personal Measurement Assistant (Model PMA2100, Solar Light Company, Inc. Philadelphia, PA) or equivalent.

Irradiation Deflector

Aluminum foil and 3 mm sheet of plate glass (if needed, large enough to cover UVA-exposed animals to limit UVB exposure during UVA exposure as necessary) or equivalent.

Patching Materials

Webril® cotton pad with adhesive tape, 2 × 2 cm or 25 mm Hilltop® Chamber (Hilltop Research, Inc., Cincinnati, OH); rubber dental dam; Blenderm® tape (Medical Surgical Division/3M, St. Paul, MN).

Animal Species

Hartley albino guinea pig

Procedure

Unless the irritation potential of the test substance is known, the study should begin with a topical range-finding study.

Optimally, the test article concentration used for induction should produce no systemic toxicity and a mild-to-moderate dermal response (grades \pm , 1 or 2) unless the test article is not dermally irritating at 100%. The test article concentration may be varied during the induction period depending on the dermal responses produced. The test article concentration(s) used for challenge should produce no systemic toxicity and dermal responses generally consisting of grades 0 to \pm unless the test article is not dermally irritating at 100%. If the results of the challenge procedure are not conclusive, then a rechallenge may need to be performed to help clarify the challenge responses. The test article concentration(s) used for rechallenge should produce no systemic toxicity and dermal responses generally consisting of grades 0 to \pm unless the test article is not dermally irritating at 100%.

Following challenge (or rechallenge), irritation scores for UVA-exposed and non-UVA-exposed sites in the test group animals are compared to the respective irritation scores for the challenge (or rechallenge) control animals. In general, a positive contact sensitization response will be considered to have occurred if the score for the non-UVA-exposed site on the test area is at least one grade higher than the highest score observed on the non-UVA-exposed site on the challenge control animals. A positive photosensitization response will be considered to have occurred for the test animals if the irritation score at the UVA-exposed site on the test animals is at least one grade higher than the highest score observed for (1) UVA-exposed site of the challenge control animals, and (2) non-UVA-exposed sites of the test group animals.

Topical Range-Finding Study

The range-finding study should include eight Hartley-derived albino guinea pigs (four males and four females). Up to four graded concentrations of the test substance may be used in this procedure. On the day before dose administration, the eight guinea pigs should be weighed and the hair removed from the left and right sides of each animal using a small animal clipper. Care should be taken to avoid abrading the skin during the clipping procedure. On the day of dose administration, a 0.3 or 0.4 mL dose of the appropriate concentration of the test substance should be administered to a 25 mm Hilltop Chamber or Webril patch, and the patch immediately

placed on the right or left side of the guinea pig (one patch on either side of the backbone). Four patches (two patches per concentration) may be applied. Immediately after application, the animals should be placed in a Buehler restrainer and the patches occluded using a rubber dental dam. The dental dam should be pulled taut over the back of the animal and fastened to the bottom of the restrainer. After an exposure period of 2 h, a 2 × 2 cm² should be cut into the dental dam and the patch removed from the right side of each animal. The patch on the left side of the animal should remain intact. The back of each animal should then be covered with an aluminum foil. An approximately 2 × 2 cm² section should be cut in the aluminum foil on each animal to expose the test site on the right side. The treated sites on the right side of the animal should then be exposed to UVA light (320–400 nm) at a target dose of 10 J/cm². Any heavy residual test substance is removed with dry gauze before irradiation to fully expose the test site. After the exposure, the foil, dental dam, and the remaining patches from the left side should be removed and any residual test substance removed with an appropriate vehicle. The dermal test sites should be graded at approximately 24 and 48 h after the initiation of the UVA light exposure.

Induction

The induction phase of the study is initiated by weighing the animals and clipping the hair from the scapular area of the 10 test and 10 challenge control animals. Care is taken to avoid abrading the skin during the clipping procedure. On the day of dose administration (day 0), four 0.1 mL intradermal injections of a 1:1 v/vFCA emulsion are administered to the previously prepared animals. The injections should be made on each side of the backbone. The center portion of the skin between the injection sites is then tape-stripped using an adhesive tape to remove the outer layers of the epidermis. A single 25 mm Hilltop® Chamber or Webril patch containing 0.3 or 0.4 mL of the test substance for the test animals and 0.3 or 0.4 mL of vehicle for the challenge control animals should be applied immediately to the center of the tape-stripped area. A piece of rubber dental dam should be placed over the application site and secured to the bottom of the restrainer to provide an occlusive binding. After approximately 2 h of exposure, a 2 × 2 cm² will be cut in the rubber dam and the patch removed. Aluminum foil is then placed over the entire back of each guinea pig, and an approximately 2 × 2 cm² window over the test area is cut to allow exposure to the UVA treatment. Any heavy residual test substance is removed with dry gauze before irradiation to fully expose the test site. The test sites should then be exposed to UVA light (320–400 nm) at a target dose of 10 J/cm². A UVB light measurement will also be recorded at the time of UVA measurements. If necessary, a glass filter or appropriate device may be used to limit the delivered UVB to 0.1 J/cm². After the completion of the exposure period, the aluminum foil and dental dam should be removed and the test substances removed with an appropriate vehicle.

The induction procedure should be repeated three times a week (e.g., Monday, Wednesday, Friday) for two consecutive weeks for a total of six induction exposures. If a positive control group is necessary, Musk Ambrette is an acceptable positive control substance, and a positive control group consisting of 10 Musk Ambrette test animals and 10 Musk Ambrette control animals should be treated in the same manner as the photosensitization study test and challenge control animals throughout the study. The Musk Ambrette concentrations standardly used for induction and challenge are 15% w/v and 0.5% w/v, respectively.

Challenge

A challenge procedure should be performed on study day 25 (± 1 day). On the day before challenge dose administration, the test and challenge control animals should be weighed and the hair removed from the left and right sides of the animal using a small animal clipper. On the next day, a 0.3 or 0.4 mL volume of the test substance should be applied to each of the two 25 mm Hilltop Chamber or Webril patches. One patch will be applied to each side of each animal. Immediately after the patching procedure, the animals should once again be placed into restrainers and the test sites immediately occluded with a piece of rubber dental dam. After approximately 2 h of exposure, a 2×2 cm² should be cut into the dental dam and the patch from the right side of each animal removed. Aluminum foil

should then be placed over the back of each animal and a 2×2 cm² window cut in the foil to allow the test area to be exposed to the UVA light. Any heavy residual test substance is removed with dry gauze before irradiation to fully expose the test site. The test sites on the right side should then be exposed to UVA light (320–400 nm) at a target dose of 10 J/cm². A UVB light measurement will also be recorded at the time of UVA measurements. If necessary, a glass filter or appropriate device may be used to limit the delivered UVB to 0.1 J/cm². After the target exposure, the foil, dental dam, and patches should be removed and any residual test substance removed with an appropriate vehicle. The test sites should be graded at approximately 24 and 48 h after the initiation of the UVA exposure using a Draize grading system.

Rechallenge

If the results of the challenge phase are not conclusive, a rechallenge procedure can be performed on study day 32 (± 1 day). The rechallenge phase should be similar in design to the challenge phase except that 10 naive rechallenge control animals and a naive skin site should be utilized for this phase.

COMPARISON OF SCORING SYSTEMS (TABLES 3.8 THROUGH 3.16)

TABLE 3.8
Draize Dermal Irritation Scoring System

Erythema and Eschar Formation	Value	Edema Formation	Value
No erythema	0	No edema	0
Very slight erythema (barely perceptible)	1	Very slight edema (barely perceptible)	1
Well-defined erythema	2	Slight edema (edges of area well-defined by definite raising)	2
Moderate-to-severe erythema	3	Moderate edema (raised approximately 1 mm)	3
Severe erythema (beet redness) to slight, eschar formation (injuries in depth)	4	Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Source: Draize, J.H., *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*, The Association of Food and Drug Officials of the United States, Austin, TX, p. 49, 1959.

TABLE 3.9
Human Patch Test Dermal Irritation Scoring System

Skin Reaction	Value
No sign of inflammation; normal skin	0
Glazed appearance of the sites, or barely perceptible erythema	$\pm (0.5)$
Slight erythema	1
Moderate erythema, possible with barely perceptible edema at the margin; papules may be present	2
Moderate erythema, with generalized edema	3
Severe erythema with severe edema, with or without vesicles	4
Severe reaction spread beyond the area of the patch	5

Source: Patrick, E. and Maibach, H.I., *Dermatotoxicology*, in *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, 1989.

TABLE 3.10
Chamber Scarification Dermal Irritation Scoring System

Skin Reaction	Value
Scratch marks barely visible	0
Erythema confined to scratches perceptible erythema	1
Broader bands of increased erythema, with or without rows of vesicles, pustules, or erosions	2
Severe erythema with partial confluency, with or without other lesions	3
Confluent, severe erythema sometimes associated with edema, necrosis, or bullae	4

Source: Patrick, E. and Maibach, H.I., *Dermatotoxicology*, in *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, 1989.

TABLE 3.11
Magnusson Sensitization Scoring System

Skin Reaction	Value
No reaction	0
Scattered reaction	1
Moderate and diffuse reaction	2
Intense reddening and swelling	3

Source: Magnusson, B. and Kligman, A., *Allergic Contact Dermatitis in the Guinea Pigs*, C.C. Thomas, Springfield, IL, 1970.

TABLE 3.12
Split Adjuvant Sensitization Scoring System

Skin Reaction	Value
Normal skin	0
Very faint, nonconfluent pink	±
Faint pink	+
Pale pink to pink, slight edema	++
Pink, moderate edema	+++
Pink and thickened	++++
Bright pink, markedly thickened	+++++

Source: Klecak, G., Identification of contact allergies: Predictive tests in animals, in *Dermatotoxicology*, 2nd edn., Marzulli, F.N. and Maibach, H.I., Eds., Hemisphere Publishing, Washington, DC, 1983.

TABLE 3.13
Buehler Sensitization Scoring System

Skin Reaction	Value
No reaction	0
Very faint erythema, usually confluent	±(0.5)
Faint erythema, usually confluent	1
Moderate erythema	2
Strong erythema, with or without edema	3

Source: Buehler, E.V. and Griffin, F., *Animal Models Dermatol.*, 55, 1975.

TABLE 3.14
Contact Photosensitization Scoring System

Skin Reaction	Value
No erythema	0
Minimal but definite erythema confluent	1
Moderate erythema	2
Considerable erythema	3
Maximal erythema	4

Source: Harber, L.C. et al., Immunologically mediated contact photosensitivity in guinea pigs, in *Dermatotoxicology*, 2nd edn., Marzulli, F.N. and Maibach, H.I., Eds., Hemisphere Publishing, Washington, DC, 1983.

TABLE 3.15
Human Patch Test Sensitization Scoring System

Skin Reaction	Value
Doubtful reaction; faint erythema only	? or + ?
Weak positive reaction; erythema, infiltration, discrete papules	+
Strong positive reaction: erythema, infiltration, papules, vesicles	++
Extreme positive reaction; intense erythema, infiltration, and coalescing vesicles	+++
Negative reaction	—
Irritant reaction of different types	IR
Not tested	NT

Source: Fischer, T. and Maibach, H.I., Patch testing in allergic contact dermatitis, in *Exogenous Dermatoses: Environmental Dermatitis*, Menné, T. and Maibach, H.I., Eds., CRC Press, Boca Raton, FL, 1991.

TABLE 3.16
Local Lymph Node Ear Scoring System

Skin Reaction	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate-to-severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

Sources: Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 429: Skin Sensitization: Local Lymph Node Assay, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442A: Skin Sensitization: Local Lymph Node Assay: DA, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, 2010.

COMPARISON OF CLASSIFICATION
SYSTEMS (TABLES 3.17 THROUGH 3.30)

TABLE 3.17
Environmental Protection Agency (EPA) Method of
Calculating the Primary Irritation Index (PII) for
Dermal Irritation Studies

Option 1

Separately add up each animal’s erythema and edema scores for the 1, 24, 48, and 72 h scoring intervals. Add all six values together and divide by the (number of test sites × 4 scoring intervals).

Option 2

Add the 1, 24, 48, and 72 h erythema and edema scores for all animals and divide by the (number of test sites × 4 scoring intervals).

Sources: U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Series 815 Dermal Irritation, 55e, 1984; U.S. Environmental Protection Agency, *Toxic Substances Control Act, Test Guidelines*, 40 CFR Part 798, Subpart E—Specific Organ/Tissue Toxicity, Section 798.4470 Primary Dermal Irritation, 491, 1992.

TABLE 3.18
Federal Hazardous Substances Act (CPSC/FHSA)
Method of Calculating the Primary Irritation Index (PII)
for Dermal Irritation Studies

Option 1

Separately add up each animal’s intact and abraded erythema and edema scores for the 25 and 72 h scoring intervals. Add all six values together and divide by the (number of test sites × 2 scoring intervals).

Option 2

Add the 25 and 72 h erythema and edema scores for all animals (intact and abraded sites) and divide by the (number of test sites × 2 scoring intervals).

Source: U.S. Consumer Products Safety Commission, 16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.3: Definitions, 381, September 2012.

TABLE 3.19
European Economic Community’s (EEC) Method
of Calculating the Primary Irritation Index (PII)
for Dermal Irritation Studies

For Six Animals

1. *Erythema*: Add all 24, 48, and 72 h erythema scores for each animal together and divide by the (number of test sites × 3 scoring intervals).
2. *Edema*: Add all 24, 48, and 72 h edema scores for each animal together and divide by the (number of test sites × 3 scoring intervals).

For Three Animals

1. *Erythema*: Add all 24, 48, and 72 h erythema scores of each animal individually and divide by the number of scoring intervals.
2. *Edema*: Add all 24, 48, and 72 h edema scores of each animal individually and divide by the number of scoring intervals.

Source: The Commission of the European Communities, Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.4, Acute Toxicity (Skin Irritation), 2008.

TABLE 3.20
Environmental Protection Agency (EPA) Dermal
Classification System

Primary Irritation Index	Irritation Rating
0.00	Nonirritant
0.01–1.99	Slight irritant
2.00–5.00	Moderate irritant
5.01–8.00	Severe irritant

Source: U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Subdivision F: Hazard Evaluation: Humans and Domestic Animals—Addendum 3 on Data Reporting, 1988.

TABLE 3.21
Environmental Protection Agency (EPA) Standard
Evaluation Procedure Dermal Classification System

Mean Score (Primary Irritation Index)	Response Category
0–0.4	Negligible
0.5–1.9	Slight
2–4.9	Moderate
5–8.0	Strong (primary irritant)

Source: U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Hazard Evaluation Division, Standard Evaluation Procedure, Guidance for Evaluation of Dermal Irritation Testing, 1, 1984.

TABLE 3.22
Federal Fungicide, Insecticide, and Rodenticide
Act (EPAFIFRA) Dermal Classification System

Toxicity Category	Warning Label
I	Corrosive. Causes eye and skin damage (or irritation). Do not get in eyes, on skin, or on clothing. Wear goggles or face shield and gloves when handling. Harmful or fatal if swallowed. (Appropriate first aid statement required.)
II	Severe irritation at 72 h. Causes eye (and skin) irritation. Do not get on skin or on clothing. Harmful if swallowed. (Appropriate first aid statement required.)
III	Moderate irritation at 72 h. Avoid contact with skin, eyes, or clothing. In case of contact, immediately flush eyes or skin with plenty of water. Get medical attention if irritation persists.
IV	Mild or slight irritation at 72 h. (No precautionary statements required.)

Source: U.S. Environmental Protection Agency, *Toxic Substances Control Act, Test Guidelines*, 40 CFR Chapter 1 (7-1-93), Part 156: Labeling Requirements for Pesticides and Devices, Section 156.10, 75, 1993.

TABLE 3.23
European Economic Community (EEC) Dermal
Classification System

Mean Erythema Score	Irritation Rating
0.00–1.99	Nonirritant
≥2.00	Irritant
Mean Edema Score	Irritation Rating
0.00–1.99	Nonirritant
≥2.00	Irritant

Source: The Commission of the European Communities, Official Journal of the European Communities, Annex VI, General Classification and Labelling Requirements for Dangerous Substances, No. L 257/11, 1983.

TABLE 3.24
Federal Hazardous Substances Act (CPSC/FHSA)
Dermal Classification System

Primary Irritation Score	Irritation Rating
0.00–4.99	Nonirritant
≥5.00	Irritant

Source: U.S. Consumer Products Safety Commission, 16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.3: Definitions, 381, September 2012.

TABLE 3.25
Draize Dermal Classification System

Primary Irritation Index	Irritation Rating
<2	Mildly irritating
2–5	Moderately irritating
>5	Severely irritating

Source: Patrick, E. and Maibach, H.I., *Dermatotoxicology*, in *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, 1989.

TABLE 3.26

Department of Transportation (DOT), Occupational Safety and Health Administration (OSHA), and International Maritime Organization (IMO) Packing Group Classification System

Corrosive Subcategories (OHSA)/Packing Categories (DOT)

	Definition
1A/I	Materials that cause full-thickness destruction of intact skin tissue within an observation period of up to 60 min starting after the exposure time of 3 min or less.
1B/II	Materials other than those meeting Packing Group I criteria that cause full-thickness destruction of intact skin tissue within an observation period of up to 14 days starting after the exposure time of more than 3 min but not more than 1 h.
1C/III	Materials, other than those meeting Packing Group I or II criteria <ol style="list-style-type: none"> 1. That cause full-thickness destruction of intact skin tissue within an observation period of up to 14 days starting after the exposure time of more than 1 h but not more than 4 h; or 2. That do not cause full-thickness tissue destruction of intact skin tissue but exhibit a corrosion rate on steel or aluminum surfaces exceeding 6.25 mm (0.25 in.)/year at a test temperature of 55°C (130°F).

Source: U.S. Research and Special Programs Administration, Department of Transportation, 49 CFR, Part 173. 136 and 137, September 2012; International Maritime Dangerous Goods Code, Class 8 Corrosives, International Maritime Organization, London, U.K., 1994; U.S. Occupational Safety and Health Administration, Labor, 29 CFR Chapter XVII, Part 1910, Appendix A to Section 1900.1200—Health Hazard Definitions (Mandatory), 2012.

Note: Several of these agencies accept human and *in vitro* testing based on OECD guidelines in some cases.

TABLE 3.27

Maximization Sensitization Classification System

Sensitization Rate, %	Grade	Classification
0	—	Nonsensitizer
>0–8	I	Weak sensitizer
9–28	II	Mild sensitizer
29–64	III	Moderate sensitizer
65–80	IV	Strong sensitizer
81–100	V	Extreme sensitizer

Source: Magnusson, B. and Kligman, A., *Allergic Contact Dermatitis in the Guinea Pigs*, C.C. Thomas, Springfield, IL, 1970.

TABLE 3.28

Optimization Sensitization Classification System

Intradermal Positive Animals, %	Epidermal Positive Animals, %	Classification
s, >75	And/or s, >50	Strong sensitizer
s, 50–75	And/or s, 30–50	Moderate sensitizer
s, 30–50	n.s., 0–30	Weak sensitizer
n.s., 0–30	n.s., 0	No sensitizer

Source: Patrick, E. and Maibach, H.I., *Dermatotoxicology*, in *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, 1989.

Notes: s, significant; n.s., not significant (using Fisher's Exact Test).

TABLE 3.29

OECD AND EPA Sensitization Classification Systems

Modified Buehler and Standard	≥15% = a mild-to-moderate sensitizer (e.g., HCA) or ≥30% = a moderate-to-strong sensitizer
Buehler tests (BTs)	(e.g., DNCB)
GPMT	≥30% = a mild-to-moderate sensitizer (e.g., HCA)

Source: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2600: Skin Sensitization Study, 2003; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 406: Skin Sensitization, 1992.

Note: Dermal scores of 1 in both the test and control animals are generally considered equivocal unless a higher dermal response is noted in the test animals.

TABLE 3.30

Local Lymph Node Classification Systems

EPA 2600 and OECD 429 (Radiolabeled Assay)	SI > 3 (SI > 20 is considered excessive)
OECD 442A (DA Method)	SI > 1.8 (1.8–2.5 considered borderline; SI > 10 is considered excessive)
OECD 442B (BrdU ELISA Method)	SI > 1.6 (1–1.9 considered borderline; SI > 14 is considered excessive)

Sources: U.S. Environmental Protection Agency, Health Effects Guidelines, OPPTS 870.2600: Skin Sensitization Study, 2003; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 429: Skin Sensitization: Local Lymph Node Assay, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442A: Skin Sensitization: Local Lymph Node Assay: DA, 2010; Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, 2010.

Note: SI, mean result from test group/mean result from vehicle control group. For borderline results, consider dose–response relationship, statistical differences, and consistency of vehicle control responses.

MATERIALS THAT PRODUCE DERMAL IRRITATION AND/OR SENSITIZATION (TABLES 3.31 THROUGH 3.33)

TABLE 3.31
Common Materials Used as Positive Controls

Material	CAS No.	Suggested Concentrations	Category
Sodium lauryl sulfate (SLS)	151-21-3	1.0%	Irritant
Hexylcinnamaldehyde (HCA)	101-86-0	25% in acetone:olive oil (4:1, v/v) (OECD 429/OECD 442A/OECD 442B)	Mild-to-moderate sensitizer
Mercaptobenzothiazole	149-30-4	5% in N,N-dimethylformamide (OECD 429)	Mild-to-moderate sensitizer
Benzocaine	94-09-7	—	Mild-to-moderate sensitizer
p-Phenylenediamine	106-50-3	—	Sensitizer
2,4-Dinitrochlorobenzene (DNCB)	97-00-7	Induction: 0.1%–0.5%, 0.25% w/v in ethanol/acetone Challenge: 0.1%–0.3%, w/v in ethanol/acetone	Sensitizer
Eugenol	97-53-0	25% in acetone:olive oil (4:1, v/v) (OECD 442A/OECD 442B)	Sensitizer
Potassium dichromate	7778-50-9	—	Sensitizer
Neomycin sulfate	1405-10-3	—	Sensitizer
Nickel sulfate	7786-81-4	—	Sensitizer
8-Methoxypsoralen (Oxsoralen Lotion®)	298-81-7	1.0%	Photoirritant
5-Methoxypsoralen (Bergapten)	298-81-7	1.0%	Photoirritant
2,4-Dinitro,3-methyl,6-tertiarybutyl-anisole (musk ambrette)	83-66-9	Induction: 10.0% w/v in ethanol/acetone Challenge: 0.5% w/v in ethanol/acetone	Photosensitizer
2-Chloro 10[3-dimethylaminopropyl] phenothiazine hydrochloride (chloropromazine)	50-53-3	Induction: 1.0% w/v in methanol Challenge: 0.1% w/v in methanol	Photosensitizer
3,3,4,5-Tetrachlorosalicylandide (TCSA)	1154-59-2	Induction: 1.0% w/v in acetone Challenge: 1.0% w/v in acetone	Photosensitizer (in mice and guinea pigs), possible sensitizer in guinea pigs

Sources: Organization for Economic Cooperation and Development, *OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 406: Skin Sensitization*, 1992; The Commission of the European Communities, *Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity*, No. L 141/142, B.6: Skin Sensitization, 2008; Charles River Laboratories, Inc., *Protocol for a Primary Irritation Study in Rabbits*, Spencerville, OH, 2012; Hakim, R.E. et al., Experimental toxicologic studies on 8-methoxypsoralen in animals exposed to the long ultraviolet, *J. Pharmacol. Exp. Ther.*, 131, 394, 1961; Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Rabbits with Non-Occlusive Conditions*, Spencerville, OH, 2012; Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Rabbits with Occlusive Conditions*, Spencerville, OH, 2012; Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Guinea Pigs with Non-Occlusive Conditions*, Spencerville, OH, 2012; Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Guinea Pigs with Occlusive Conditions*, Spencerville, OH, 2012; Gad, S.C. and Chengelis, C.P., *Acute Toxicol. Test.*, 117, 1990; Buehler, E.V. and Griffin, F., *Animal Models Dermatol.*, 55, 1975; Charles River Laboratories, Inc., *Protocol for a Dermal Sensitization Study in Guinea Pigs—Modified Buehler Design*, Spencerville, OH, 2000; Buehler, E.V., *Arch. Dermatol.*, 91, 171, 1965; Charles River Laboratories, Inc., *Protocol for a Dermal Sensitization Study in Guinea Pigs—Standard Buehler Design*, Spencerville, OH, 2000; Magnusson, B. and Kligman, A., *Allergic Contact Dermatitis in the Guinea Pigs*, C.C. Thomas, Springfield, IL, 1970; Charles River Laboratories, Inc., *Protocol for a Dermal Sensitization Study in Guinea Pigs—Maximization Design*, Spencerville, OH, 2000; Gerberick, G.F. et al., The local lymph node assay ICCVAM test method submission, April, 1998, in *The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds*, NIH Publication No. 99–4494, February 1999; Siglin, J.C. et al., *Evaluation of a New Murine Model for the Predictive Assessment of Contact Photoallergy (CPA)*, American College of Toxicology Annual Meeting, Savannah, GA, 1991; Charles River Laboratories, Inc., *Protocol for a Photoallergy Study in Mice*, Spencerville, OH, 1994; Ichikawa, H. et al., *J. Invest. Dermatol.*, 76, 498, 1981; Charles River Laboratories, Inc., *Protocol for a Photosensitization Study in Guinea Pigs*, Spencerville, OH, 2000.

TABLE 3.32
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Dyes	Clothing and Textiles			
Aminoazotoluene		✓		
Anthraquinones dyes (Disperse blue 35)		✓	✓	
Azo dyes		✓		
Chromium dyes		✓		
Disperse yellow 39 (methene dye)		✓		
Naphthol AS (azo dye)	✓			
p-Phenylenediamine (PPD)		✓		
Resins				
Dimethyl oldihydroxyethylene		✓		
Dimethyl olethylene urea		✓		
Dimethyl urea (urea formaldehyde)		✓		
Formaldehyde		✓		
Melamine formaldehyde		✓		
Adhesives				
Dodecyl mercaptan	✓	✓		
p-tertiary-butylphenol formaldehyde (PTBP resin)		✓		
Rubber boots				
Isopropylaminodiphenylamine (IPPD)		✓		
Mercaptobenzothiazole (MBT)		✓		
Fiberglass	✓			
	Cosmetics			
Aldehyde citronellal		✓		
Aluminum palls (in deodorants)	✓			
Ammonium persulfate		✓		
Balsam Peru		✓		✓
Benzoyl salicylate		✓		
Celaltronium chloride		✓		
Chloro-3,5-xyleneol 4-(chloroxylenol)	✓	✓		
Cinnamic acid		✓		
Cinnamic aldehyde		✓		
di-tert-butyl hydrogunone		✓		
dl- a-tocopherol		✓		
Henna		✓		
Lanolin		✓		
Lemongrass oil		✓		
Perfume		✓		
Propellants in deodorant		✓		
Sorbitan monostearate	✓	✓		
Sorbitan monooleate	✓	✓		
Triethanolamine	✓			
Zerconium		✓		
	Foods			
Artichoke		✓		
Asparagus		✓		
Carrot		✓		✓
Cheese		✓		
Chives		✓		
Cucumber		✓		
Endive		✓		
Fish		✓		
Flour		✓		
Garlic		✓		

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Horseradish		✓		
Leek		✓		
Lemon peel		✓		
Lettuce		✓		
Meats		✓		
Onion		✓		
Poultry skin and flesh		✓		
Shellfish		✓		
Wheat flour		✓		
Vanilla		✓		
Food Additives				
4-Hydroxybenzoic acid		✓		
Ammonium and potassium persulfates		✓		
Butylated hydroxyanisole		✓		
Butylated hydroxytoluene		✓		
Dodecyl gallate	✓			
Ethoxyquin				✓
Hydroquinone		✓		✓
Monosodium glutamate		✓		
Octyl gallate	✓			
Propyl gallate		✓		
Sodium benzoate		✓		
Sorbic acid		✓		
Sulfur dioxide		✓		
Medicants				
Ampicillin		✓		
Antihistamines		✓		
Benadryl		✓		
Benzoic acid		✓		
Benzophenone		✓		
Benzoyl peroxide		✓		
Coumarin	✓			✓
Dimercaprol		✓		
Estrogen cream		✓		
Fluorouracil		✓		✓
Gentian violet		✓		
Hydrocortisone		✓		✓
Mafenide acetate		✓		✓
Monoamyl amine		✓		
Neomycin sulfate		✓		
Oxyphenbutazone		✓		
p-Chlorobenzene sulfonyl glycolic acid nitrile		✓		
Penicillin		✓		
Pristinamycin		✓		
Promethazene hydrochloride		✓		✓
Quinoderm		✓		
Retinoic acid		✓		
Salicylic acid		✓		
Streptomycin		✓		
Sulisobenzone		✓		✓
Sulfonamides		✓		
Tetracycline (also phototoxic)		✓		

(continued)

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Metals				
Arsenic	✓			
Beryllium salts		✓		
Cadmium sulfide				✓
Chromate		✓		
Nickel		✓		
Selenium	✓			
Pesticides				
Dichlorophene	✓			
Dinitrochlorobenzene		✓		
Dinobuton	✓			
Diothiocarbarnates		✓		
Lindane		✓		
Malathion		✓		
Maneb		✓		
Omite	✓			
Radox		✓		
Zineb		✓		
Plants				
Angelica	✓			
Anise	✓			
Boneset		✓		
Burdock		✓		
Caraway	✓			
Celeriac	✓			
Celery	✓			
Chamomile		✓		
Cocklebur		✓		
Coriander	✓			
Cow parsley	✓			
Daffodil, narcissus		✓		
Dill	✓			
Fennel	✓			
Feverfew		✓		
Giant hogweed	✓			
Hogweed, cow parsnip	✓			
Ivy		✓		
Marshelder		✓		
Masterwort	✓			
Poison ivy and oak		✓		
Poverty weed		✓		
Primula		✓		
Pyrethrum, tansy		✓		
Ragweed		✓		
Ragweed of florists and species (alantolactone and parthenium)		✓		
Sage brush/wormwood		✓		
Sneezeweed (alantolactone and parthenium)		✓		
Tansy		✓		
Tulip		✓		
Fruits and Vegetables				
Artichoke		✓		
Brussels sprouts, cabbage		✓		
Carrot	✓	✓		

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Celery		✓		
Chicory, endive		✓		
Chive, leek, onion, garlic		✓		
Horseradish		✓		
Lettuce		✓		
Orange, lemon, lime		✓		
Parsley	✓	✓		
Parsnip	✓	✓		
Pineapple		✓		
Woods				
Abura	✓			
African blackwood	✓			
African mahogany	✓			
American mahogany	✓			
Australian blackwood	✓			
Ayan	✓			
Camphor	✓	✓		
Cassia oil		✓		
Ceylon satinwood	✓			
Cocobolo	✓			
Cocus	✓			
Common alder	✓			
Douglas fir	✓			
English elm	✓			
Gaboon	✓			
Grevillea	✓			
Ipe	✓			
Iroko	✓			
Limba	✓			
Louro	✓			
Macassar ebony	✓			
Makore	✓			
Mansonia	✓			
Opepe	✓			
Peroba rosa	✓			
Pine oil	✓			
Ramin	✓			
Teak	✓			
Toporite	✓			
Western red cedar	✓			
White peroba	✓			
White poplar	✓			
West Indian satinwood	✓			
Yew	✓			
Plastics				
Acrylamide		✓		
Acrylonitrile		✓		
Cyanoacrylic acids and esters	✓			
Diacylates (delayed)	✓			
Methacrylonitrile (poison)		✓		
Methyl, ethyl, and n-butyl methacrylates		✓		
Epoxy Resin Systems				
Allyl resin — diallylglycol carbonate	✓			
Diallylphthalate	✓			
Dimethylaniline (poison)	✓			

(continued)

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Diphenylmethane diisocyanate	✓			
Epoxy monomer		✓		
Hardener		✓		
Hexamethylene diisocyanate	✓			
Maleic acid anhydride	✓			
Naphthalene diisocyanate		✓		
Naphthoquinone (poison)	✓			
Peroxides (catalyst)	✓			
Phthalic acid anhydride	✓			
p-tert-Butyl phenol formaldehyde (PTBP)		✓		
Reactive diluent		✓		
Toluene diisocyanate	✓			
Hardeners				
Isophoronediamine		✓		
N-Aminoethylpiperazine		✓		
Polyether alcohol		✓		
Polyurethane laquar	✓			
Triethylamine	✓			
Cellulose Polymers				
Cellophane		✓		
Celluloid		✓		
Cellulose nitrate		✓		
Collodion		✓		
Gun cotton		✓		
Pyroxylin		✓		
Rayon		✓		
Regenerated cellulose		✓		
Antioxidant: p-tert-butyl phenol		✓		
Colors: azo dyes solvent yellow 3, solvent red 26, pigment red 481		✓		
Components: plastizer, triphenyl phosphate		✓		
Polish turpentine		✓		
Solvent: ethylene glycol monomethyl ether acetate		✓		
Ultraviolet light stabilizer: resorcinol monobenzoate		✓		
Preservatives and Antibacterials				
Preparations containing Lanolins (I)/Parabins (P)/Chlorocresol(c)				
Adcortyl cream (p)		✓		
Betnovate cream (c)		✓		
Betnovate lotion (p)		✓		
Cortenema (p)		✓		
Dermovate cream (c)		✓		
Efcortelan cream (c)		✓		
Efcortelan lotion (p)		✓		
Hydrocortistab eye ointment (1)		✓		
Hydromycin-Dornlucet ear/eye (1)		✓		
Medrone acne lotion (p)		✓		
Medrone cream (p)		✓		
Motivate cream (c)		✓		
Myciguent ointment (1)		✓		
Myciguent ophthalmic ointment (1)		✓		
Neo-Cortef eye and ear ointment (1)		✓		
Neo-Cortef ointment (p)		✓		
Neo-Cortef lotion (p)		✓		

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Neo-Medione acne lotion (p)		✓		
Nerisone cream (p)		✓		
Nystadermal cream (p)		✓		
Nystadermal gel (c)		✓		
Propaderm cream (c)		✓		
Propaderm lotion (c)		✓		
Remiderm cream (p)		✓		
Schericur ointment (l)		✓		
Synolar creams except for Synalar Forti cream (p)		✓		
Synolar combination creams (p)		✓		
Synolar ointments (l)		✓		
Topilar ointment (l)		✓		
Topisone (l)		✓		
Triadcortyl cream (p)		✓		
Ultradil cream plain (p)		✓		
Ultradil ointment plain (l)		✓		
Ultralanum cream plain (l)		✓		
Ultralanum lotion (p)		✓		
Ultralanum ointment (l)		✓		
Ultralanum ointment plain (l)		✓		
Phenolic compounds				
Hexachlorophane		✓		
Mercury				
Ammoniated mercury		✓		
Mercuric chloride	✓	✓		
Mercurochrome	✓			
Mercury fulminate (mercuric cyanate)	✓			
Merthiolate	✓			
Phenylmercuric acetate	✓	?		
Phenylmercuric borate		✓		
Phenylmercuric nitrate		✓		
Phenylmercuric propionate		✓		
Quaternary ammonium compounds				
Benzalkonium chloride	✓			
Bronopol	✓			
Cetalkonium chloride		✓		
Cetrimide		✓		
Chlorhexidine		✓		
Chloroacetamide		✓		
Ethylene oxide	✓			
Rubber/Latex Components				
Thiurams				
Dipentamethylenethiuram disulfide		✓		
Tetraethylthiuram disulfide		✓		
Tetramethylthiuram disulfide		✓		
Tetramethylthiuram monosulfide		✓		
Mercapto Group				
Cyclohexylbenzothiazylsulfenamide		✓		
Dibenzothiazyl disulfide		✓		
Mercaptobenzothiazole		✓		
Morpholinylmercaptobenzothiazole		✓		
PPD Group				
Diaminodiphenylmethane		✓		
Diphenyl-PPD		✓		

(continued)

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Isopropylphenyl-PPD (isopropylamino diphenylamine)		✓		
Phenylcyclohexyl-PPD		✓		
Naphthyl Group				
Phenyl- P-naphthylamine		✓		
sym-Di-P-naphthyl-PPD		✓		
Carbamates				
Zinc diethyldithiocarbamate		✓		
Zinc dibutyldithiocarbamate		✓		
Miscellaneous				
Dioxydiphenyl		✓		
Diphenylguanidine		✓		
Dithiodimorpholine		✓		
Miscellaneous Compounds				
Acetaldehyde (10%)		✓		
Acetyl-1,1,2,3,3,6-hexamethylindan				✓
Acetylacetone (slightly)		✓		
Acridine				✓
Acriflavine				✓
Alcohol, anhydrous		✓		
Allyl butyrate (4%)	✓			
Allyl cinnamate (0.1%)	✓			
Allyl cyclohexylacetate	✓			
Allyl epoxypropoxybenzene		✓		
Allyl phenylacetate	✓	✓		
Aminobenzoic acid derivatives			✓	
Aminobenzoic acid p–				✓
Aminophenol o– and p–		✓		
Aminosalicyclic acid p–				✓
Aminothiazole		✓		
Aminodarone			✓	
Ammonia		✓		
Amyl dimethylamino benzoate, mixed ortho and para isomers			✓	
Amyl dimethyl PABA				✓
Amyl nitrite	✓			
Amyl phenylacetate J	✓			
Anthracene-acridine			✓	
Atranorin				✓
Benzaldehyde		✓		
Benzylamine hydrochloride				✓
Bergamot oil				✓
Bergapten (5-methoxypsoralen)			✓	
Bromomethyl-4-nitrobenzene		✓		
Buclosamide				✓
Butylphenol		✓		
Cadmium chloride				✓
Cadmium sulfate				✓
Caraway oil				✓
Carbimazole				✓
Carotene P-				✓
Cephalosporins		✓		
Cetyl alcohol		✓		
Chlor-2-phenylphenol				✓
Chloramine-T		✓		

TABLE 3.32 (continued)**Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization**

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Chlormercaptodicarboximide				✓
Chloro-6-fluorobenzaldehyde-a-chlorooxime2-		✓		
Chlorodiazepoxide			✓	
Chlorothalonil	✓			
Chlorothiazides			✓	✓
Chlorpromazine				✓
Cinoxate				✓
Cinnamon bark oil Ceylon				✓
Citral		✓		
Clobetasol-17-propionate		✓		
Coal tar			✓	✓
Cobalt chlorate				✓
Cobaltous chloride		✓		
Cobaltous chlorate				✓
Cobaltous nitrate				✓
Cobaltous sulfate				✓
Cocamide DEA	✓			
Cocamphocarboxyglycinate		✓		
Coniferyl benzoate		✓		
Cu(II)-acetyl acetate		✓		
Cumin oil				✓
Cyanamide		✓		
Dacarbazine			✓	
Decylaminoethanethiol 2-n-		✓		
Deneclocycline			✓	
Dexpanthenol	✓			
Diamine N-		✓		
Diaminodiphenylmethane		✓		✓
Dibucain hydrochloride				✓
Dichloro-2-phenylphenol				✓
Dichloroquinoline		✓		
Dicyclohexylcarbodiimide		✓		
Diethazine				✓
Diethylaminopropylamine		✓		
Diethyl fumarate		✓		
Diethylstilbestrol				✓
Digalloyl trioleate				✓
Diglycidyl ether		✓		
Dihydrocoumarin		✓		
Dimethoxane				✓
Dimethyl antranilate				✓
Dimethyl sulfoxide		✓		
Diethyl-p-phenylenediamine		✓		
Diphenhydramine hydrochloride				✓
Diphenylcyclopropanone		✓		
Diphenyl-p-phenylenediamine		✓		
Dipyrene		✓		
Docusate sodium	✓			
Erythrosine				✓
Ethacridine lactate monohydrate				✓
Ethyl aminobenzoate		✓		✓
Ethyl ether	✓			
Ethylparaben		✓		
Fig leaf absolute				✓

(continued)

TABLE 3.32 (continued)

Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Furocoumarins			✓	
Geraniol		✓		
Geranyl formate	✓			
Ginger oil				✓
Griseofulvin			✓	✓
Glyceryl p-aminobenzoate				✓
Halogenated phenols				
Bithionol				✓
Bromochlorosalicylanilide (Multifungin)				✓
Chlorophenylphenol				✓
Dibromosalicylanilide (DBS)				✓
Fentichlor				✓
Hexachlorophene Buclosamide (Jadit)				✓
Tetrachlorosalicylanilide 3, 3', 4', 5 (TCSA)				✓
Tribromosalicylanilide (TBSA)				✓
Trichlorcarbanilide (TCCA)				✓
Hexanediol diacrylate	✓			✓
Hexantriol		✓		
Hydratropic aldehyde	✓			
Iothion		✓		
Isoamyl alcohol		✓		
Isocamphyl cyclohexanol	✓			
Isopropyl alcohol		✓		
Isostearoamphopropionate		✓		
Ketoprofen				✓
Kynuremic acid			✓	
Lauroamphocarboxyglycinate		✓		
Lauroamphoglycinate		✓		
Lauroamphopropionate		✓		
Lauryl isoquinolinium bromide	✓			
Lavender oil				✓
Mannide monooleate	✓			
Mechlorethamine hydrochloride (nitrogen mustard)		✓		
Menthol 1-		✓		
Mepazine				✓
Metamizole		✓		
Methoxyethylepoxypropoxybenzene		✓		
Methylanisalacetone a-	✓			
Methylcoumarin 6-				✓
Methylcoumarin 7-				✓
Methylene blue				✓
Methylisothiazolinone		✓		
Methylparaben		✓		
Methyl salicylate		✓		
Minoxidil				✓
Musk ambrette	✓			
Musk xylol				✓
Myrcanodin				✓
Neosilversalvarsan		✓		
Neroli oil				✓
Neutral Red		✓	✓	
Nicotinyl alcohol		✓		
Nitrofuroxime		✓		
Nitrose dimethyl aniline		✓		
Nonoxynol-9 y	✓			
Oak moss				✓

TABLE 3.32 (continued)**Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization**

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Octoxynol-9 y	✓			
Oleamide		✓		
Oxybenzone				✓
Padimate A or Escalol 506 (amyl p-dimethylaminobenzoate)			✓	
Papain		✓		
PBA-1				✓
Pelargonic acid y	✓			
Pentadecylcatechol 3-		✓		
Pentamethyl-4,6-dinitroindane 1, 1, 3, 3, 5- y	✓			✓
Pentanol		✓		✓
Pentylidenecyclohexanone		✓		
Perphenazine				✓
Petitgrain oil Paraguay				✓
Phenanthrene				✓
Phenol		✓		
Phenothiazine				✓
Phenylacetaldehyde		✓		
Phenylbenzimidazol sulfate 2-				✓
Phenyl butazone		✓		
Phenyl glycidyl ether				✓
Phenylphenol				✓
Phosphorus sesquisulfide		✓		
Picryl chloride		✓		
Pigment orange 5				✓
Pigment orange red 49, calcium lake				✓
Pinus pumilio oil	✓			
Pitch			✓	✓
Platinum salts		✓		
Polysorbate 20	✓			
Polysorbate 60		✓		
Polysorbate 80	✓			
Primin		✓		
Prochlorperazine			✓	
Promazine				✓
Propionaldehyde		✓		
Propyl alcohol n—		✓		
Propylphenbutazone		✓		
Psoralens			✓	
Pyridine				✓
Pyridoxine hydrochloride		✓		
Pyrilamine maleate				✓
Quinine				✓
Quinine hydrochloride				✓
Quinine sulfate				✓
Quinoline methanol			✓	
Rhodamine B				✓
Ricinoleic acid	✓			
Rose Bengal				✓
Rue oil				✓
Silver bromide				✓
Silver fulminate	✓			
Silver nitrate				✓
Sodium hypochlorite	✓			

(continued)

TABLE 3.32 (continued)
Materials Categorized by Their Ability to Produce Dermal Irritation or Sensitization

Material	Irritant	Sensitizer	Photoirritant	Photosensitizer
Sodium monoglyceride sulfide	✓			
Sodium monoglyceride sulfonate	✓			
Sodium octoxynol-2 ethane sulfonate	✓			
Sodium oleyl laurate	✓			
Sodium stearate	✓			
Sodium sulfide	✓	✓		
Sodium thiosulfate	✓			
Sorbitan laurate	✓	✓		
Squaric acid-diethylester		✓		
Stearalkonium chloride	✓			
Stearoamphoglycinate		✓		
Stearyl alcohol		✓		
Stictic acid				✓
Stilbene triazine				✓
Sulbentine				✓
Sulfadiazine				✓
Sulfamerazine				✓
Sulfamethazine				✓
Sulfanilamide			✓	✓
Sulfathiazole				✓
Sulfur		✓		
Sulisobenzene				✓
Thioridazine				✓
Thiourea				✓
Thurfyl nicotinate		✓		
Toluidine red				✓
Tribomsalan				✓
Tributyltin oxide	✓			
Trichlorosalicylanilide 3',4',5-				✓
Triclocarban				✓
Triethylenemelamine				✓
Trimeprazine				✓
Trinitrobenzene sym-		✓		
Tropicamide		✓		
Turkey-red oil	✓			✓
Umbelliferone				✓
Undecylenic aldehyde digeranyl acetal	✓			
Valeraldehyde		✓		
Vetiverol	✓			
Vinyl pyridine 4-		✓		
Xanthotoxin (8-methoxypsoralen)			✓	
Zinc pyrithione			✓	

Sources: Kimber, I. et al., *J. Toxicol. Environ. Health*, A, 52, 563, 1998; Gerberick, G.F. and Ryan, C.A., *Food Chem. Toxicol.*, 27, 813, 1989; Maurer, T., Predictive animal test methods for allergenicity, in *Contact and Photocontact Allergens, A Manual of Predictive Test Methods*, Vol. 3, Calnan, C.D. and Maibach, H.I., Eds., Marcel Dekker, New York, 1983; U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Hazard Evaluation Division, Standard Evaluation Procedure, Guidance for Evaluation of Dermal Irritation Testing, 1, 1984; U.S. Environmental Protection Agency, *Toxic Substances Control Act, Test Guidelines*, 40 CFR Chapter 1 (7-1-93), Part 156: Labeling Requirements for Pesticides and Devices, Section 156.10, 75, 1993.

TABLE 3.33
Dermal Irritants and Sensitizers Listed by Occupation

Occupation	Irritant	Sensitizer
Agricultural workers	Artificial fertilizers Disinfectants and cleansers for milking machines Petrol and diesel oil	Rubber (clothing and milking equipment)
		Oats
		Barley
		Animal feed (antibiotics, preservatives, additives, and cobalt)
		Veterinary medicaments
		Cement
		Plants
		Pesticides
		Wood preservatives
		Turpentine
		Pigments (cobalt, nickel, and chromium)
		Azo dyes
		Anthraquinone dyes
Artists and sculptors	Solvents Clay Plaster	Aminoazotoluene
		Colophony
		Epoxy resin
		Chromate (primers, passivators, anticorrosives, welding fumes, oils)
Automobile and aircraft mechanics	Solvents Cutting oils Paints Hand cleansers	Nickel
		Cobalt
		Rubber
		Epoxy resins
		Dimethacrylate resins
		Dipentene in thinners
		Flavors and spices (cinnamon, eugenol, vanilla, cardamom)
		Orange
		Lemon
		Lime
Bakers and confectioners	Flour detergents	Pineapple
		Essential oils
		Dyes
		Ammonium persulfate
		Benzoyl peroxide (improvers in flour)
		Orange
		Lemon
		Lime
		Flavors
		<i>o</i> -Phenylphenol (in some detergents)
Book binders	Solvents Glues	Glues
		Resins
		Leather
Butchers	Detergents Meat Offal	Meat (contact urticaria)
		Teak (knife handles)
		Nickel
		Sawdust
Cabinet makers, French polishers, carpenters	Detergents Solvents Thinners for cleaning metal (as a cause of koilonychia, Ancona Alayon, 1975) Wood and wood preservatives	Stains (including dichromate)
		Glues (urea, phenol, PTBP formaldehyde resins)
		Wood
		Turpentine
		Varnishes
		Colophony

(continued)

TABLE 3.33 (continued)
Dermal Irritants and Sensitizers Listed by Occupation

Occupation	Irritant	Sensitizer
Cable jointers	Solvents	Epoxy resin Fluxes (aminoethylethanolamine)
Cleaners	Detergents Solvents Wet work	Rubber gloves Chromates (bleaches in some countries)
Coal miners	Dust (coal, stone) Cement Wet conditions	Rubber boots Masks
Construction workers	Cement	Chromate Cobalt Gloves (rubber, leather) Resins (epoxy and formaldehyde) Woods
Cooks and catering	Detergents Food juices Wet work Parsley Parsnip Carrots	Foods (contact urticaria) Onion Garlic Lettuce Carrots Celery Parsley Parsnip Brussels sprouts Cabbage Spices Flavors Rubber gloves Sodium metabisulfite Lauryl Octyl gallate Formaldehyde (deodorizing solution, fishmongers)
Dentists and dental technicians	Detergents Hand cleaners Wet work	Local anesthetics (amethocaine, procaine) Methacrylates Eugenol (eugenol and colophony gingivectomy dressing) Mercury Disinfectants Rubber Dental impression material (Impregum and Scutan: the sensitizers are the catalysts methyldichlorobenzene sulfonate and methyl <i>p</i> -toluoyl sulfonate)
Dry cleaners	Solvents	Rubber gloves
Electricians	Soldering fluxes	Fluxes (colophony, hydrazine) Insulating tape (colophony) Resins (epoxy and formaldehyde) Rubber
Electroplaters	Acids Alkalis	Nickel Chromium Other metals Rubber gloves
Embalmers and morticians	Disinfectants Detergents	Formaldehyde
Floor layers	Solvents	Cement Resins (epoxy and formaldehyde) Woods Varnish Linoleum (colophony)

TABLE 3.33 (continued)
Dermal Irritants and Sensitizers Listed by Occupation

Occupation	Irritant	Sensitizer
Florists	Manure Fertilizers Pesticides Wet work	Plants (alantolactone and parthenium) Pesticides (DNCB, dichlorophene, lindane) Rubber gloves
Foundry workers	Cleansers	Phenol and urea formaldehyde (resincoated sand) Colophony (nitrogen-free sand) Gloves (rubber, chromium)
Funeral directors		Floral tributes (alantolactone and parthenium)
Garage workers	Petroleum products Diesel fuel Cleansers Detergents Solvents	Rubber gloves Chromate Epoxy resin Antifreeze (MBT)
Gardeners	Artificial fertilizers	Pesticides Plants/flowers Rubber gloves Boots
Hairdressers	Shampoos Perming solutions Bleaching solutions Wet work	Dyes (<i>p</i> -phenylenediamine, <i>p</i> -toluoylenediamine, <i>o</i> -nitro <i>p</i> -phenylenediamine, <i>p</i> -aminiphenol, henna) Persulfates Rubber gloves Lanolin Perfumes Lemongrass oil Formaldehyde (shampoos) Resorcinol Pyrogallol Nickel
Hospital workers	Detergents Disinfectants Foods Wet work	Rubber gloves Disinfectants Flowers Foods Polishes Hand creams
Housework	Detergents Cleaners Foods Disinfectants Wet work	Rubber gloves Foods (onions, garlic, citrus fruit; contact urticaria) Spices Flavors Hand creams Nickel Chromate (bleaches) Flowers Polishes
Jewelers	Detergents Solvents	Epoxy resin Metals (nickel, chromium) Sawdust (used for drying jewelry)
Metal workers	Cutting and drilling oils Solvents Hand cleansers	Chromates Additives in cutting oils (antibacterials and antioxidants)
Nurses	Disinfectants Detergents Wet work	Rubber gloves Formaldehyde Glutaraldehyde

(continued)

TABLE 3.33 (continued)
Dermal Irritants and Sensitizers Listed by Occupation

Occupation	Irritant	Sensitizer
Office workers	Photocopying (ammonia)	Dettol
		Disinfectants
		Medicaments (including antibiotics, antihistamines, hydrocortisone, retinoic acid, chlorpromazine)
		Flowers
		Rubber (finger stalls)
		Nickel (clips, photocopying solutions)
		Copy papers
		Carbon papers
		Correction paper fluids
		Turpentine
Painters	Solvents Thinners Wallpaper adhesives Hand cleansers	Dipentene
		Cobalt (driers, colors)
		Chromate (colors)
		Wallpaper adhesives (formaldehyde, chloroacetamide, and fungicides)
		Paints (preservatives, e.g., mercurials)
Photograph developers (x-ray technicians)	Wet work Solvents	Rubber gloves
		<i>p</i> -Aminophenol (Metol)
		Color developers
		Hydroquinone
		Phenindone
		Sodium metabisulfite
		EDTA
		Glutaraldehyde
		Pyrogallol
		Amidol
		Ethylenediamine
		Resorcinol
		Triazine
		Salicylaldehyde
		Monomers
Plastic industry	Solvents Acids Styrene Oxidizing agents Hardeners (polyurethane lacquer, triethylamine)	Hardeners (isophoronediamine, polyether alcohol)
		Additives
		Cellulose polymers
		Cellulose acetate
		Epoxy resin systems
Plating industry	Acids Alkalis Solvents	Nickel
		Chromate
		Cobalt
Plumbers	Wet work Cleaners	Mercury
		Chromate (cement)
Printers	Solvents	Rubber (gloves, packing)
		Chromate
		UV-cured inks
		Colophony (paper)
		Turpentine
		Rubber gloves
		Rubber blanket in offset printing
		Formaldehyde (gum arabic)
		Resins (epoxy)
		Fluxes (colophony and hydrazine)
Radio and television workers	Fluxes	Chromate
		Rubber chemicals
		Dyes
		Colophony
Rubber workers	Solvents Talc Zinc stearate	

TABLE 3.33 (continued)
Dermal Irritants and Sensitizers Listed by Occupation

Occupation	Irritant	Sensitizer
Secretaries		Carbon paper Photocopy paper (azo compound, thiourea photosensitizer) Correcting paper Rubber (fingerstall and rubber bands) Glues (PTBP resin, colophony) Leather Rubber Turpentine
Shoemakers and cobblers	Solvents	Tanning agents (chromium, vegetable tans, glutaraldehyde, formaldehyde) Rubber (gloves and boots) Fungicides Dyes Formaldehyde resins
Tannery workers	Acids Alkalis Reducing and oxidizing agents Wet work	Dyes Chromate (mordant) Nickel
Textile workers	Fibers Bleaching agents Solvents	Rubber gloves Medicaments used to treat animals and which contaminate their fur Medicaments Tuberculin Benethamate Benzylpenicillin Spiramycin Tylosin Penethamete Neomycin in a calf drench Mercaptobenzothiazole in a medication Benzisothiazolone fungicide Topical pesticides; malathion Contact urticaria from animal tissues Cow hair and dander in bacon factories, workers eviscerating or cleaning the guts develop an eczema of the fingers, known as "gut" or "fat" eczema; its cause is unknown
Veterinarians (and slaughterhouse workers)	Disinfectants Wet work Entrails Animal secretions	Lichens (atranorin) Glues Varnishes Colophony Turpentine Balsams
Wood workers	Woods	

Sources: Gerberick, G.F. and Ryan, C.A., *Food Chem. Toxicol.*, 27, 813, 1989; Maurer, T., Predictive animal test methods for allergenicity, in *Contact and Photocontact Allergens, A Manual of Predictive Test Methods*, Vol. 3, Calnan, C.D. and Maibach, H.I., Eds., Marcel Dekker, New York, 1983; Fischer, T. and Maibach, H.I., Patch testing in allergic contact dermatitis, in *Exogenous Dermatoses: Environmental Dermatitis*, Menné, T. and Maibach, H.I., Eds., CRC Press, Boca Raton, FL, 1991; DeGroot, A.C., *Patch Testing, Test Concentrations and Vehicles for 2800 Allergens*, Elsevier Science, Amsterdam, the Netherlands, 1986; Cronin, E., *Contact Dermatitis*, Churchill Livingstone, New York, 1980.

REFERENCES

1. U.S. Environmental Protection Agency, *Health Effects Guidelines, OPPTS 870.2500*, Acute Dermal Irritation, August, 1998.
2. Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Irritation Studies (2-1-4), 13-14, November, 2000.
3. Organization for Economic Cooperation and Development, *OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 404: Acute Dermal Irritation/Corrosion*, 1, 2002.
4. The Commission of the European Communities, *Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.4. Acute Toxicity (Skin Irritation)*, 2008.

5. U.S. Environmental Protection Agency, *Health Effects Guidelines, OPPTS 870.2600: Skin Sensitization Study*, 2003.
6. Japan New Drugs Division Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, 1990 *Guidelines for Toxicity Studies of Drugs Manual*, 1991, Chapter 7.
7. Japan Ministry of Agriculture, Forestry and Fisheries, Agricultural Production Bureau, The guidelines related to the study reports for the registration application of pesticide, Appendix to Director General Notification, Skin Sensitization Studies (2-1-6), November 16–18, 2000.
8. Organization for Economic Cooperation and Development, *OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 406: Skin Sensitization*, 1992.
9. The Commission of the European Communities, *Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.6: Skin Sensitization*, 2008.
10. Organization for Economic Cooperation and Development, *OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 429: Skin Sensitization: Local Lymph Node Assay*, 2010.
11. Organization for Economic Cooperation and Development, *OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442A: Skin Sensitization: Local Lymph Node Assay: DA*, 2010.
12. Organization for Economic Cooperation and Development, *OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Subsection 442B: Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA*, 2010.
13. The Commission of the European Communities, *Official Journal of the European Communities, Part B: Methods for the Determination of Toxicity, No. L 141/142, B.42: Skin Sensitization: Local Lymph Node Assay*, 2008.
14. U.S. Environmental Protection Agency, *Office of Pesticide Programs: Expansion of the Traditional Local Lymph Node Assay for the Assessment of Dermal Sensitization Potential of End Use Pesticide Products and Adoption of a "Reduced" Protocol for the Traditional LLNA (Limit Dose)*, January 27, 2011.
15. U.S. Consumer Products Safety Commission, *16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.41: Method of Testing Primary Irritant Substances*, September 2012.
16. U.S. Consumer Products Safety Commission, *16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.3: Definitions*, 382, September 2012.
17. U.S. Research and Special Programs Administration, Department of Transportation, 49 CFR, Part 173. 136 and 137, September 2012.
18. U.S. Pharmacopeia, National Formulary, *USP 24 NF 19 Biological Reactivity Test*, INVIVO, 1832, 2000, Chapter 88.
19. *A Guide to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Part 3 Health Hazards*, United Nations, 2007.
20. Canadian Environmental Protection Act, *Guidelines for the Notification and Testing of New Substances: Chemicals and Polymers, Section 5.1: Test Procedures and Practices*, 50, 1993.
21. International Maritime Dangerous Goods Code, Class 8 Corrosives, International Maritime Organization, London, 1994.
22. Occupational Safety and Health Administration, Labor, 29 CFR Chapter XVII, Part 1910, Appendix A to Section 1900.1200: Health Hazard Definitions (Mandatory), 2012.
23. American Society for Testing and Materials, *1991 Annual Book of ASTM Standards*, F719–81 (1986), 13.01: Practice for Testing Biomaterials in Rabbits for Primary Skin Irritation, 976, 1991.
24. American Society for Testing and Materials, *1991 Annual Book of ASTM Standards*, F720–81 (1986), 13.01, Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test, 976, 1991.
25. American Society for Testing and Materials, *1991 Annual Book of ASTM Standards*, E993–88, 11.01, Test Method for Evaluation of Delayed Contact Hypersensitivity, 964, 1991.
26. The Cosmetic, Toiletry and Fragrance Association, Inc., *CTFA Safety Testing Guidelines*, Section II: Guidelines for Evaluating Primary Skin Irritation Potential, 2, 1991.
27. The Cosmetic, Toiletry and Fragrance Association, Inc., *CTFA Safety Testing Guidelines*, Section IV, Guidelines for Evaluating Contact Sensitization Potential, 7, 1991.
28. Draize, J.H., *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*, The Association of Food and Drug Officials of the United States, Austin, TX, p. 49, 1959.
29. Charles River Laboratories, Inc., *Protocol for a Primary Irritation Study in Rabbits*, Spencerville, OH, 2012.
30. Charles River Laboratories, Inc., *Protocol for a Dermal Corrosivity Study in Rabbits*, Spencerville, OH, 2012.
31. Hakim, R.E., Freeman, R.G., Griffin, A.C., and Knox, J.M., Experimental toxicologic studies on 8-methoxypsoralen in animals exposed to the long ultraviolet, *J. Pharmacol. Exp. Ther.*, 131, 394, 1961.
32. OECD Guidelines for the Testing of Chemicals, Acute dermal photoirritation screening test, February, 1995 (Draft Proposal).
33. Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Rabbits with Non-Occlusive Conditions*, Spencerville, OH, 2012.
34. Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Rabbits with Occlusive Conditions*, Spencerville, OH, 2012.
35. Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Guinea Pigs with Non-Occlusive Conditions*, Spencerville, OH, 2012.
36. Charles River Laboratories, Inc., *Protocol for a Photoirritation Study in Guinea Pigs with Occlusive Conditions*, Spencerville, OH, 2012.
37. Gad, S.C. and Chengelis, C.P., Photosensitization and phototoxicity, *Acute Toxicol. Testing*, 117, 1990.
38. Buehler, E.V. and Griffin, F., Experimental skin sensitization in the guinea pig and man, *Animal Models Dermatol.*, 55, 1975.
39. Charles River Laboratories, Inc., *Protocol for a Dermal Sensitization Study in Guinea Pigs — Modified Buehler Design*, Spencerville, OH, 2000.
40. Buehler, E.V., Delayed contact hypersensitivity in the guinea pig, *Arch. Dermatol.*, 91, 171, 1965.
41. Charles River Laboratories, Inc., *Protocol for a Dermal Sensitization Study in Guinea Pigs — Standard Buehler Design*, Spencerville, OH, 2000.
42. Magnusson, B. and Kligman, A., *Allergic Contact Dermatitis in the Guinea Pigs*, C.C. Thomas, Springfield, IL, 1970.
43. Charles River Laboratories, Inc., *Protocol for a Dermal Sensitization Study in Guinea Pigs — Maximization Design*, Spencerville, OH, 2000.
44. Gerberick, G.F., Kimber, I., and Basketter, D., The local lymph node assay ICCVAM test method submission, April, 1998, in *The Murine Local Lymph Node Assay: A Test Method*

- for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds, NIH Publication No. 99-4494, February 1999.
45. Kimber, I., Hilton, J., Dearman, R., Gerberick, G.F., Ryan, C., Basketter, D.A., Lea, L. et al., Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: an interlaboratory evaluation, *J. Toxicol. Environ. Health*, A, 52, 563–579, 1998.
 46. Loveless, S.E., Ladics, G.S., Gerberick, B.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., House, R.V., Hilton, J., Dearman, R.J., and Kimber, I., Further evaluation of the local lymph node assay in the final phase of an international collaborative trial, *Toxicology*, 108, 141–152, 1996.
 47. Dearman, R.J., Hilton, J., Evans, P., Harvey, P., Basketter, D.A., and Kimber, I., Temporal stability of local lymph nodes assay response to hexylcinnamic aldehyde, *J. Appl. Toxicol.*, 18, 281–284, 1998.
 48. Siglin, J.C., Jenkins, P.K., Smith, P.S., Ryan, C.A., and Gerberick, G.F., *Evaluation of a New Murine Model for the Predictive Assessment of Contact Photoallergy (CPA)*, American College of Toxicology Annual Meeting, Savannah, GA, 1991.
 49. Gerberick, G.F. and Ryan, C.A., A predictive mouse ear-swelling model for investigating topical phototoxicity, *Food. Chem. Toxicol.*, 27, 813, 1989.
 50. Charles River Laboratories, Inc., *Protocol for a Photoallergy Study in Mice*, Spencerville, OH, 1994.
 51. Ichikawa, H., Armstrong, R.B., and Harber, L.C., Photoallergic contact dermatitis in guinea pigs: Improved induction technique using Freund's complete adjuvant, *J. Invest. Dermatol.*, 76, 498, 1981.
 52. Harber, L.C., Shalita, A.R., and Armstrong, R.B., Immunologically mediated contact photosensitivity in guinea pigs, in *Dermatotoxicology*, 2nd edn., Marzulli, F.N. and Maibach, H.I., Eds., Hemisphere Publishing, Washington, DC, 1983.
 53. Maurer, T., Predictive animal test methods for allergenicity, in *Contact and Photocontact Allergens, A Manual of Predictive Test Methods*, Vol. 3, Calnan, C.D. and Maibach, H.I., Eds., Marcel Dekker, New York, 1983.
 54. Charles River Laboratories, Inc., *Protocol for a Photosensitization Study in Guinea Pigs*, Spencerville, OH, 2000.
 55. Patrick, E. and Maibach, H.I., *Dermatotoxicology*, in *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, 1989.
 56. Klecak, G., Identification of contact allergies: predictive tests in animals, in *Dermatotoxicology*, 2nd edn., Marzulli, F.N. and Maibach, H.I., Eds., Hemisphere Publishing, Washington, DC, 1983.
 57. Fischer, T. and Maibach, H.I., Patch testing in allergic contact dermatitis, in *Exogenous Dermatoses: Environmental Dermatitis*, Menné, T. and Maibach, H.I., Eds., CRC Press, Boca Raton, FL, 1991.
 58. U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Series 815 Dermal Irritation, 55e, 1984.
 59. U.S. Environmental Protection Agency, *Toxic Substances Control Act, Test Guidelines*, 40 CFR Part 798, Subpart E—Specific Organ/Tissue Toxicity, Section 798.4470 Primary Dermal Irritation, 491, 1992.
 60. U.S. Consumer Products Safety Commission, 16 CFR Chapter II, Subchapter C: Federal Hazardous Substances Act Regulation, Part 1500, Subsection 1500.3: Definitions, 381, September 2012.
 61. U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Subdivision F: Hazard Evaluation: Humans and Domestic Animals—Addendum 3 on Data Reporting, 1988.
 62. U.S. Environmental Protection Agency, *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Hazard Evaluation Division, Standard Evaluation Procedure, Guidance for Evaluation of Dermal Irritation Testing, 1, 1984.
 63. U.S. Environmental Protection Agency, *Toxic Substances Control Act, Test Guidelines*, 40 CFR Chapter 1 (7-1-93), Part 156: Labeling Requirements for Pesticides and Devices, Section 156.10, 75, 1993.
 64. The Commission of the European Communities, Official Journal of the European Communities, Annex VI, General Classification and Labelling Requirements for Dangerous Substances, No. L 257/11, 1983.
 65. U.S. Occupational Safety and Health Administration, Labor, 29 CFR Chapter XVII, Part 1910, Appendix A to Section 1900.1200—Health Hazard Definitions (Mandatory), 2012.
 66. DeGroot, A.C., *Patch Testing, Test Concentrations and Vehicles for 2800 Allergens*, Elsevier Science, Amsterdam, the Netherlands, 1986.
 67. Cronin, E., *Contact Dermatitis*, Churchill Livingstone, New York, 1980.

APPENDIX: ADDITIONAL RELATED INFORMATION (TABLES 3.A.1 THROUGH 3.A.3)

TABLE 3.A.1
Relative Ranking of the Skin Permeability in Different Animal Species

Ranking	Animal Species	Thickness of Stratum Corneum	Epidermis (µm)	Whole Skin (mm)
Most permeable	Mouse	5.8	12.6	0.84
	Guinea pig			
	Goat			
	Rabbit			
	Horse			
	Cat			
	Dog			
	Monkey			
	Pig	26.4	65.8	3.43
	Human	16.8	46.9	2.97
Least permeable	Chimpanzee			

Source: With permission from Macmillan Publishers Ltd. Leung, H.W. and Paustenbach, D.J., Percutaneous toxicity, in *General and Applied Toxicology*, Ballantyne, B., Marrs, T.C., and Syversen, T., Eds., Groves's Dictionaries, New York, Chapter 29, pp. 577–586, Copyright 1999.

TABLE 3.A.2
***In Vivo* Human Percutaneous Absorption Rates of Some**
Neat Chemical Liquids

Chemical	Percutaneous Absorption Rate (mg cm ⁻² h ⁻¹)
Aniline	0.2–0.7
Benzene	0.24–0.4
2-Butoxyethanol	0.05–0.68
2-(2-Butoxyethoxy) ethanol	0.035
Carbon disulfide	9.7
Dimethylformamide	9.4
Ethylbenzene	22–33
2-Ethoxyethanol	0.796
2-(2Ethoxyethoxy) ethanol	0.125
Methanol	11.5
2-Methoxyethanol	2.82
Methyl <i>n</i> -butyl ketone	0.25–0.48
Nitrobenzene	2
Styrene	9–15
Toluene	14–23
Xylenes (mixed)	4.5–9.6
<i>m</i> -Xylene	0.12–0.15

Source: With permission from Macmillan Publishers Ltd. Leung, H.W. and Paustenbach, D.J., Percutaneous toxicity, in *General and Applied Toxicology*, Ballantyne, B., Marrs, T.C., and Syversen, T., Eds., Groves's Dictionaries, New York, Chapter 29, pp. 577–586, Copyright 1999.

TABLE 3.A.3
***In Vitro* Human Percutaneous Permeability Coefficients of Aqueous Solutions of Some**
Industrial Chemicals

Organic Chemical	K_p (cm h ⁻¹)	Organic Chemical	K_p (cm h ⁻¹)	Inorganic Chemical	K_p (cm h ⁻¹)
2-Amino4-nitrophenol	0.00066	2-Ethoxyethanol	0.0003	Cobalt chloride	0.0004
4-Amino2-nitrophenol	0.0028	<i>p</i> -Ethylphenol	0.035	Mercuric chloride	0.00093
Benzene	0.11	Heptanol	0.038	Nickel chloride	0.0001
<i>p</i> -Bromophenol	0.036	Hexanol	0.028	Nickel sulfate	<0.000009
Butane-2,3-diol	<0.00005	Methanol	0.0016	Silver nitrate	<0.00035
<i>n</i> -Butanol	0.0025	Methyl hydroxybenzoate	0.0091		
2-Butanone	0.0045	B-Naphthol	0.028		
Chlorocresol	0.055	3-Nitrophenol	0.0056		
<i>o</i> -Chlorophenol	0.033	4-Nitrophenol	0.0056		
<i>p</i> -Chlorophenol	0.036	Nitrosodiethanol amine	0.0000055		
Chloroxylenol	0.059	Nonanol	0.06		
<i>m</i> -Cresol	0.015	Octanol	0.061		
<i>o</i> -Cresol	0.016	Pentanol	0.006		
<i>p</i> -Cresol	0.018	Phenol	0.0082		
Decanol	0.08	Propanol	0.0017		
2,4-Dichlorophenol	0.06	Resorcinol	0.00024		
Diethanolamine	0.000034	Thymol	0.053		
Diethyl ether	0.016	Toluene	1.01		
1,4-Dioxane	0.00043	2,4,6-Trichlorophenol	0.059		
Ethanol	0.0008	3,4-Xylenol	0.036		
Ethanolamine	0.000043				

Source: With permission from Macmillan Publishers Ltd. Leung, H.W. and Paustenbach, D.J., Percutaneous toxicity, in *General and Applied Toxicology*, Ballantyne, B., Marrs, T.C., and Syversen, T., Eds., Groves's Dictionaries, New York, Chapter 29, pp. 577–586, Copyright 1999.

Note: Values obtained from viable excised human skin using a temperature-controlled skin penetration chamber.

4 Ocular Toxicology

Brendan J. Dunn, MS and Kathryn S. Monds, MS

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ANATOMY OF THE EYE

Exposure to many substances can disrupt the delicate ocular tissues, with the cornea being most likely to be affected by topical exposures and the visceral tunic and retina primarily affected by systemic agents. The ocular tissues detailed herein are those that generally demonstrate toxicological effects resulting from either topical

exposure or systemic administration of various substances. The descriptions provided in the following sections are for the human eye, unless otherwise noted. Figures 4.1 through 4.3 show anatomical structures of the human eye in sagittal section, whereas Figures 4.4 and 4.5 respectively depict the basic structures of the rabbit eye and cornea in sagittal section.

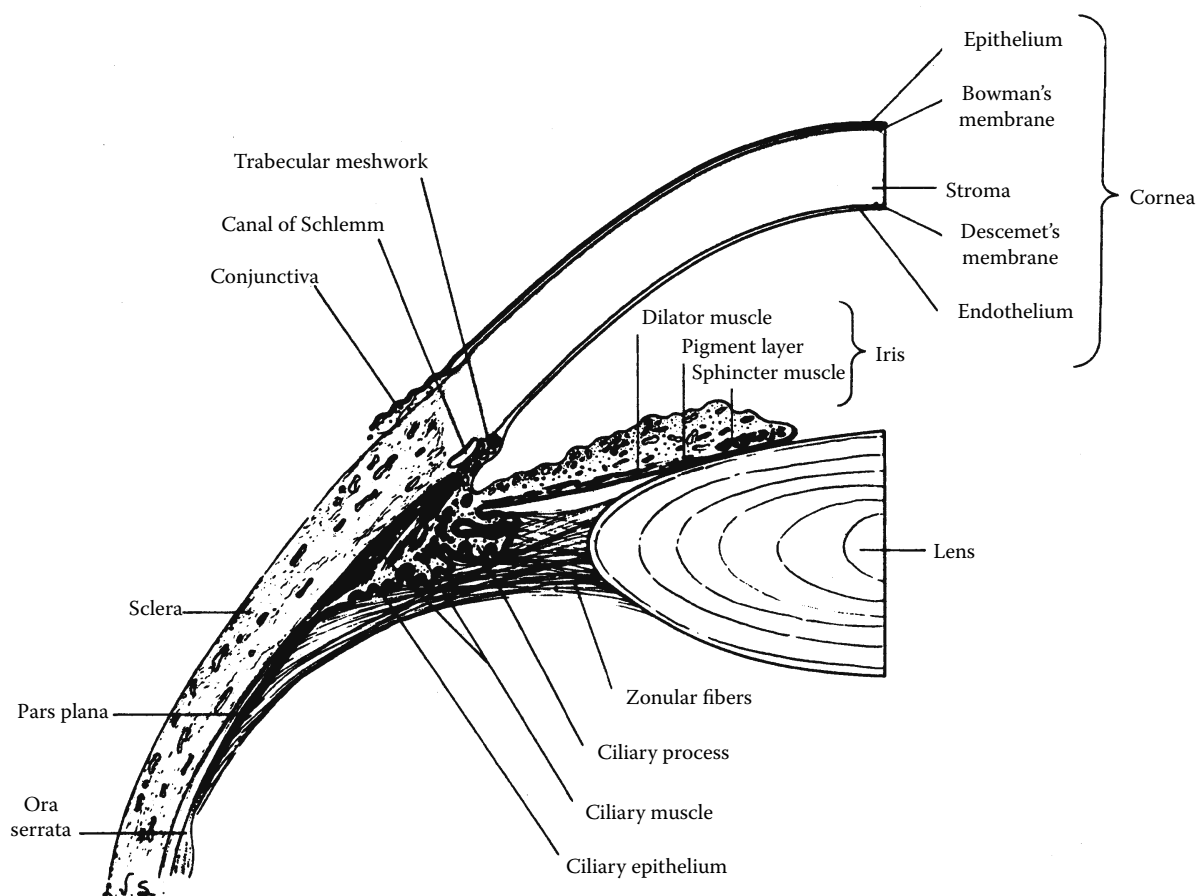


FIGURE 4.1 Sagittal section of the anterior chamber angle of the human eye showing the structure associated with the ciliary body, iris, cornea, and lens. (From Vaughan, D. et al., *General Ophthalmology*, 13th edn., Appleton & Lange, East Norwalk, CT, 1992. With permission.)

OCULAR MUCOSA

1. **Conjunctiva:** a thin, vascularized, transparent membrane covering the posterior surface of the eyelids (palpebral conjunctiva) and the anterior surface of the sclera (bulbar conjunctiva). The conjunctiva is a squamous, nonkeratinized epithelium containing numerous mucus-secreting cells.
2. **Nictitating membrane:** in the rabbit, a prominent cartilaginous flap of tissue covered by a layer of squamous epithelium that is attached in the medial canthus of the eye and moves laterally or diagonally across the eye behind the external eyelids.

OCULAR GLOBE

Fibrous Tunic

The outer tissues of the eye are composed of the transparent cornea and opaque sclera. Comprised of largely similar constituents, it is primarily the difference in organization and arrangement of collagen fibers, as well as

water content, which allows for the observed dissimilarity in transparency.

1. **Cornea:** a highly innervated, avascular, transparent tissue that occupies the anterior 1/6 of the fibrous tunic and is composed of five histologic layers:
 - a. **Epithelium:** the anterior-most corneal tissue consisting of four to six layers of nonkeratinized stratified squamous epithelial cells, which comprises approximately 10% of total corneal thickness.
 - b. **Bowman's layer or membrane:** an acellular membranous layer that serves to bridge the corneal epithelium and stroma, found only in humans, nonhuman primates, and cattle; continuous with the collagen fibers of the anterior stroma.
 - c. **Stroma:** constituting the bulk of the cornea at approximately 90% of total thickness, the stroma is composed of predominantly type I collagen fibrils (lamellae), proteoglycans, and extracellular matrix, with only 3%–5% of the stromal volume comprising keratocytes.

- d. Descemet's membrane: composed of primarily type IV collagen and laminin, secreted by but discontinuous from the endothelium, with a prenatally formed banded layer anteriorly, and a posterior nonbanded layer that thickens with age.
 - e. Endothelium: a single sheet of approximately 400,000 interdigitated endothelial cells forming a tight junction between the cornea and aqueous humor.
2. Sclera: the fibrous, opaque, outer portion of the eye that makes up 5/6 of the fibrous tunic and is responsible for maintaining the shape of the ocular globe and protection of intraocular structures.

The sclera is continuous with the cornea anteriorly and with the dural sheath of the optic nerve and lamina cribrosa posteriorly. The sclera is composed of two primary layers of collagen and elastic fibers:

- a. Episclera: the outer layer of the sclera composed of thin, elastic, and highly vascularized connective tissue lying between the superficial scleral stroma and Tenon's capsule.
- b. Lamina fusca: the loose, pigmented layer of connective tissue on the inner scleral surface that connects to the optic nerve at the choroid.

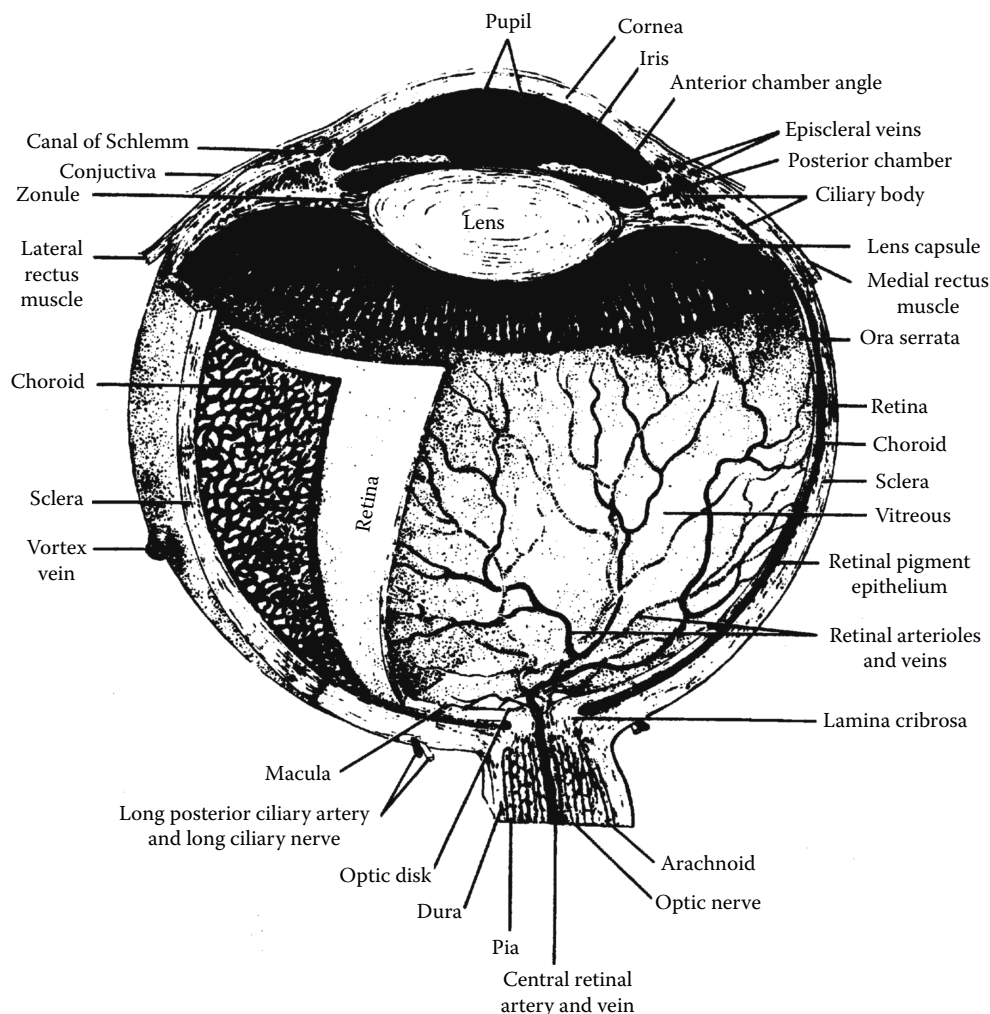


FIGURE 4.2 Sagittal section of the human eye showing internal structures. (Reprinted from *The Anatomy of the Eye*, from original drawing by Paul Peck. Copyright, Lederle Laboratories Division of American Cyanamid Company, Wayne, NJ. With permission.)

Uveal Tract

Also known as the vascular tunic, the uveal tract provides nutrients to ocular tissues, and absorbs reflected and outside light to improve retinal contrast. This pigmented middle layer of the eye is composed of the iris, the ciliary body, and the choroid.

1. Iris: a highly vascularized anterior extension of the ciliary body located in front of the lens, forming the posterior wall of the anterior chamber and the anterior wall of the posterior chamber. There are two pigmented layers on the posterior surface of the iris (except in albinos), the fibrovascular stroma and a two-cell thick epithelial layer. Within the stroma lie the sphincter and dilator muscles that determine the size of the medially located pupil, and thus the amount of light reaching the retina.
2. Ciliary body: the vascularized tissue extending forward from the anterior end of the choroid to the root of the iris. The ciliary muscle within this tissue is composed of longitudinal, circular, and radial fibers that alter the tension on the lens capsule to provide accommodation, a variable focus for both near and distant objects in the visual field.
3. Choroid: the heavily vascularized posterior segment of the uveal tract located between the retina and the sclera composed of three layers of choroidal blood vessels and Bruch's membrane. Anteriorly, it joins the ciliary body, and posteriorly it attaches to the margins of the optic nerve.

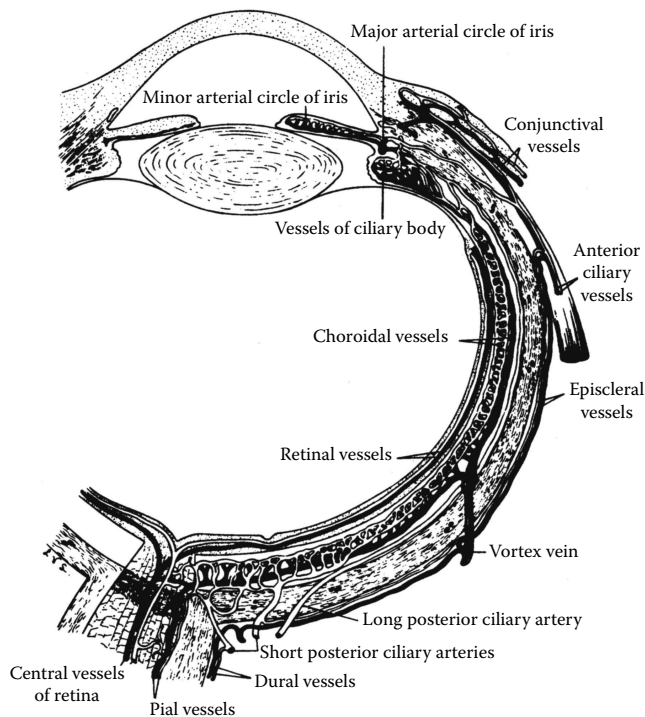


FIGURE 4.3 Sagittal section of the human eye showing the vascular supply. All arterial branches originate with the ophthalmic artery. (From Vaughan, D. et al., *General Ophthalmology*, 13th edn., Appleton & Lange, East Norwalk, CT, 1992. With permission.)

Lens

The lens is a biconvex, avascular, transparent structure suspended behind the iris by the ciliary zonule, which connects to the ciliary body. The zonule, or suspensory ligament of the lens, is composed of numerous fibrils arising from the ciliary body and inserting into the equator of the lens. The aqueous is anterior to the lens and the vitreous posterior to it. The lens is encapsulated by a semipermeable membrane, the lens capsule.

Aqueous Humor

A slightly alkaline liquid composed mainly of water, the aqueous humor is secreted by the ciliary process. It fills the anterior and posterior chambers of the eye, passing through the pupil from the posterior chamber into the anterior chamber then toward the filtering trabecular meshwork at the periphery and into the canal of Schlemm.

Vitreous Humor

A clear avascular gelatinous body filling the posterior chamber between the lens and retina, vitreous humor is comprised of 99% water, with collagen fibrils, various proteins, hyaluronic acid, inorganic salts, sugars, and ascorbic acid making up the final 1%. The vitreous comprises two thirds of the volume and weight of the eye, and aids in maintenance of the shape and transparency of the globe.

Retina

This innermost posterior coat of the eye is composed of 10 histologically distinct layers of highly organized, delicate nerve tissue. The inner surface is in contact with the vitreous, adhering only at the optic nerve disk, and the outer surface is related to the choroid. The layers of the retina are: (1) internal limiting membrane, (2) a layer of nerve fibers, (3) a ganglion cell layer, (4) inner plexiform layer, (5) inner nuclear layer, (6) outer plexiform layer, (7) outer nuclear layer, (8) external limiting membrane, (9) layer of rods and cones, and (10) pigment epithelium. Anteriorly, it extends almost as far as the ciliary body, ending in a ragged edge called the ora serrata. At the ora serrata, the nerve tissue of the retina ends, but a thin pigmented layer of the retina continues further anteriorly to relate to the posterior surfaces of the ciliary processes and the iris. In the center of the posterior segment of the retina is the macula lutea, an oval yellowish spot with a depressed center called the fovea centralis, which is responsible for central visual fields. The optic disk (the visible portion of the optic nerve) is located about 3 mm to the medial side of the macula.

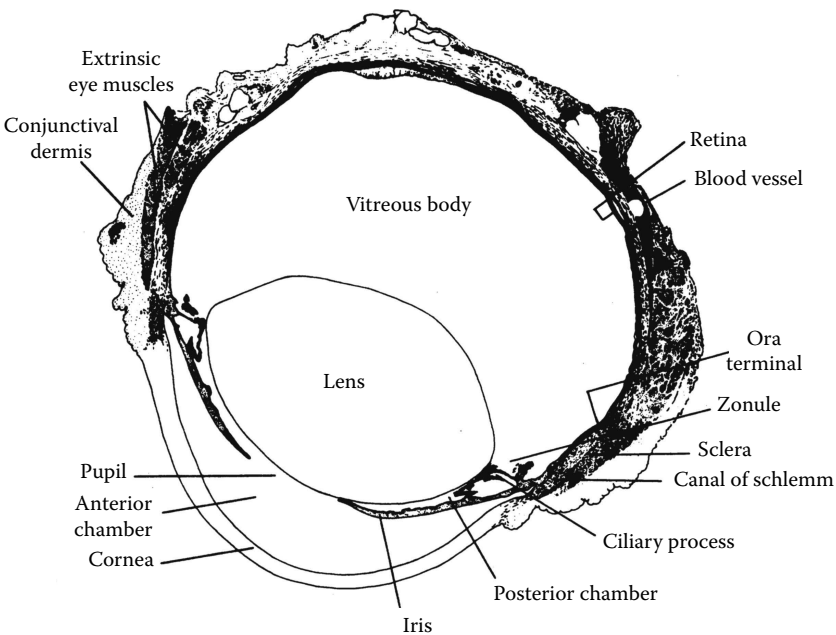


FIGURE 4.4 Sagittal section of the rabbit eye showing the basic structures. (Reprinted from McLaughlin, C.A. and Chiasson, R.B., *Laboratory Anatomy of the Rabbit*, 3rd edn., Wm. C. Brown Communications, Dubuque, IA, 1990. With permission.)



FIGURE 4.5 Sagittal section of a healthy rabbit cornea with all five histologic layers clearly visible (Courtesy of Dr. Mary Marquart, Department of Microbiology, University of Mississippi Medical Center, Jackson, MS.)

Optic Nerve

A nerve fiber tract derived from the ganglion cells of the retina, the optic nerve emerges from the posterior surface of the ocular globe through a short, circular opening in the sclera located approximately 1 mm below and 3 mm nasal

to the posterior pole of the eye. From the orbit, the optic nerve travels through the bony optic foramen into the cranial cavity where it joins the opposite optic nerve, forming the optic chiasm.

COMPARATIVE ANATOMY AND PHYSIOLOGY OF THE EYE

ANATOMICAL COMPARISONS (TABLES 4.1 THROUGH 4.4)

TABLE 4.1
Corneal Thickness and Area

Species	Thickness (mm)	Area (%) ^a	Reference
Human	0.51–0.54	7	[1,2]
Rhesus monkey	0.52	—	[3]
Rabbit	0.37–0.4	25	[1,3]
Mouse	0.1	50	[1]
Rat	0.15	50	[1]
Cat	0.62	—	[3]
Dog	0.55	—	[3]

^a Percentage of the total area of the globe.

TABLE 4.2
Comparison of the Type of Retinal Vasculature of Various Species

Species	Holangiatic	Merangiatic	Paurangiatic	Anangiatic
Pig				
Dog	×			
Cat	×			
Human	×			
Primate	×			
Rabbit		×		
Rat	×			
Mouse	×			
Gerbil	×			
Cattle	×			
Horse			×	
Guinea pig				×
Chinchilla				×
Degu				×
Bird				×

Source: Hobson, D.W. *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

Note: Holangiatic: The retinal blood supply is from a central retinal or cilioretinal arteries and extends over the entire retina. Merangiatic: A portion of the retina is supplied by retinal vessels. Paurangiatic: Retinal vessels are small and extend only a very short distance from the optic nerve. Anangiatic: The retina is without vessels.

TABLE 4.3
Percentage of Optic Nerve Fibers Decussating at the Optic Chiasm

Species	Decussation (%)
Human	50
Primate	50
Dog	75
Cat	65–70
Horse	81
Cow	83
Pig	88
Bird	100

Source: Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

TABLE 4.4
Comparison of the Tapetum in the Cat, Dog, and Ferret

Parameter	Dog	Cat	Ferret
Number of central cell layers	9	16–20	7–10
Thickness of central tapetum	26–33 μm	61–67 μm	23–24 μm
Presence of microtubule-like structure in tapetal rod	Present	Absent	Present
Presence of electron-dense cores in tapetal rod	Absent	Present	Absent
Presence of electron-dense cores in tapetal rods after prolonged glutaraldehyde fixation	Absent	Present	Absent
Retention of tapetal color after prolonged glutaraldehyde fixation	Lost	Retained	Lost
Tapetal zinc concentration	26,000 ppm	1497 ppm	22,500 ppm
Tapetal cysteine concentration	241 $\mu\text{mol/g}$	0	216 $\mu\text{mol/g}$

Sources: Modified from Wen, G.Y. et al., *Lab. Anim. Sci.*, 35, 200, 1985; Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

PHYSIOLOGICAL COMPARISONS (TABLES 4.5 THROUGH 4.8)

TABLE 4.5
Concentrations of the Principal Components of the Aqueous Humor as Compared with Plasma of Various Species

Substance and Units	Aqueous	Plasma	Species	Ref.
Ascorbate				
$\mu\text{mol/mL}$	1.18	0.02	Monkey	4
	0.96	0.02	Rabbit	5
	1.06	0.04	Human	6
mg/dL	20.0		Horse	7
	5.5	—	Dog	7
	1.0	—	Cat	7
	21.0	—	Cow	7
Bicarbonate				
$\mu\text{mol/mL}$	22.5	18.8	Monkey	4
	27.7	24.0	Rabbit	5
	20.2	27.5	Human	6
mm/g H_2O	Ratio of aqueous/plasma	0.82	Horse	7
	Ratio of aqueous/plasma	1.13	Dog	7
	30.4	25.3	Cat	8
	36.0	—	Cow	8

(continued)

TABLE 4.5 (continued)
Concentrations of the Principal Components
of the Aqueous Humor as Compared with Plasma
of Various Species

Substance and Units	Aqueous	Plasma	Species	Ref.
Calcium				
μmol/mL	2.5	4.9	Monkey	9
	1.7	2.6	Rabbit	8
	—	—	Human	
mEq/L	3.0	5.5	Horse	7
	2.9	5.24	Dog	8
	2.7	4.8	Cat	8
	—	—	Cow	7
Chloride				
μmol/mL	—	—	Monkey	
	105.1	111.8	Rabbit	10
	131.0	107.0	Human	6
mEq/L	12.1	10.1	Horse	7
	Ratio of aqueous/ plasma	1.07	Dog	7
	—	—	Cat	
	Ratio of aqueous/ plasma	1.15	Cow	8
Glucose				
μmol/mL	3.0	4.1	Monkey	5
	4.9	5.3	Rabbit	10
	2.8	5.9	Human	6
mg/dL	98	91	Horse	7
	51	70	Dog	7
	45	56	Cat	7
	33	57	Cow	7
Hyaluronate				
μmol/mL	—	—	Monkey	
	—	—	Rabbit	
	1.1	—	Human	11
	—	—	Horse	
	—	—	Dog	
	—	—	Cat	
	4.4	—	Cow	11
Lactate				
μmol/mL	4.3	3.0	Monkey	4
	9.3	10.3	Rabbit	10
	4.5	1.9	Human	6
	—	—	Horse	
	—	—	Dog	
	—	—	Cat	
	—	—	Cow	
Oxygen				
mm Hg	—	—	Monkey	
	30	77	Rabbit	12
	53	—	Human	13
	—	—	Horse	
	45	—	Dog	8
	—	—	Cat	
	—	—	Cow	
Phosphate				
μmol/mL	0.14	0.68	Monkey	5
	0.89	1.49	Rabbit	10

TABLE 4.5 (continued)
Concentrations of the Principal Components
of the Aqueous Humor as Compared with Plasma
of Various Species

Substance and Units	Aqueous	Plasma	Species	Ref.
	0.62	1.11	Human	14
	0.33	0.31	Horse	15
	0.53	1.26	Dog	7
	0.48	1.87	Cat	7
	—	—	Cow	
Potassium				
μmol/mL	3.9	4.0	Monkey	4
	5.1	5.6	Rabbit	10
	—	—	Human	
mEq/AL	5.1	5.5	Horse	15
	5.0	4.4	Dog	8
	4.4	4.0	Cat	8
	7.1	4.7	Cow	8
Protein				
mg/100 mL	33.3	—	Monkey	4
	25.9	—	Rabbit	16
	23.7	—	Human	16
	20.0	730	Horse	15
	38.0	650	Dog	17
	15–55	780	Cat	17
	17.0	750	Cow	15
Sodium				
μmol/mL	152	148	Monkey	4
	143	146	Rabbit	10
	—	—	Human	
	117.4	143.5	Horse	15
	149.4	154	Dog	8
	158.5	163.6	Cat	8
	149.5	143	Cow	7
Urea				
μmol/mL	6.1	7.3	Monkey	4
	7.0	9.1	Rabbit	8
	—	—	Human	
mg/dL	28	27	Horse	15
	Ratio of aqueous/ plasma	0.70	Dog	7
	Ratio of aqueous/ plasma	0.73	Cat	7
	—	12	Cow	7
Creatinine				
μmol/mL	0.04	0.03	Monkey	4
	0.11	—	Rabbit	18
	—	—	Human	
	0.18	0.18	Horse	15
	—	—	Dog	
	—	—	Cat	
	—	—	Cow	

Sources: Modified from Schmidt, G.M. and Coulter, D.B., *Veterinary Ophthalmology*, Gelatt, K.N., Ed., Lea & Febiger, Philadelphia, PA, 129, 1981; Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

TABLE 4.6
Concentration of the Various Components
of the Vitreous

Constituent	Cattle	Rabbit	Pig	Human	Horse
Inorganic constituents (mmol/kg H₂O)					
Sodium	130.5	133.9–152.2	142	137	118–153
Potassium	7.7	5.1–10.2	5.0	3.8	4.9–7.3
Calcium	3.9	1.5	5.7		4.9–7.3
Magnesium	0.8	—	2.6		—
Chloride	115.6	104.3	118	112.8	112–120
Water and organic constituents (mg/100 mL H₂O)					
Creatinine	1.0	—	0.5	—	—
Water	99%	99%	99%	99%	99%
Glucose	55–62	55–80	—	30–70	57–100
Lactic acid	14.8	65		70	17.5

Sources: Modified from Nordmann, J., *Biologie et Chirurgie du Corps Vitre*, Brini, A., Ed., Masson et Cie, Paris, France, 1968; Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission; Graymore, C.N., *Biochemistry of the Eye*, Academic Press, New York, 1970; McLaughlin, P.S. and McLaughlin, B.G., Chemical analysis of bovine and porcine vitreous humors: Correlation of normal values with serum chemical values and changes with time and temperature, *Am. J. Vet. Res.*, 48, 467, 1987.

TABLE 4.7
Concentration of Mucopolysaccharide and Collagen
of the Vitreous in Various Species

Species	Mucopolysaccharide (μg/mL)	Collagen (μg/mL)
Rabbit	31	104
Guinea pig	37	134
Human	240	286
Owl monkey	423	25
Steer	710	57

Sources: Modified from Gloor, B.P., *Adler's: Physiology of the Eye*, 8th edn., Moses, R.A. and Hart, W.H., Eds., C.V. Mosby, St. Louis, p. 246, 1987; Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

TABLE 4.8
Distribution of the Anterior Uveal Adrenergic
Receptors in Various Species

	Dilator	Sphincter	Ciliary Muscle
Cat	Mainly alpha, some beta	Mainly beta, some alpha	Mainly beta, some alpha
Rabbit	Mainly alpha, few beta	Mainly beta, few alpha	Mainly alpha, few beta
Monkey	Mainly alpha, very few beta	Mainly alpha, perhaps beta	Exclusively beta, no alpha
Man	Mainly alpha, very few beta	Alpha and beta in equal amounts	Mainly beta, very few or no alpha

Sources: Modified from Van Alphen, G.W., *Invest. Ophthalmol. Visual Sci.*, 15, 502, 1976; Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

REGULATORY GUIDELINES

CONTACT LENS MATERIALS

At a minimum, the following toxicology test procedures are recommended for contact lens materials by the Premarket Notification [510(k)] Guidance Document for Daily Contact Lenses. This guidance document was last revised in May 1994 by the Contact Lens Branch, Division of Ophthalmic Devices, Center for Devices and Radiological Health, Food and Drug Administration (FDA). The toxicology studies are generally consistent with the applicable studies recommended for evaluating plastic polymers in the Tripartite Biocompatibility Guidance for Medical Devices, which categorize contact lenses as externally communicating devices: intact natural channels. The Tripartite Guidance has been harmonized with the International Standards Series ISO 10993, Biological Evaluation of Medical Devices.

Systemic Injection Test (USP/NF)*

This test assesses the potential of leachable chemical constituents from a contact lens material to produce an acute systemic toxicity in mice. Extracts of the lens material are prepared in two types of solvents (polar and nonpolar), injected into mice, and the mice observed for acute systemic toxicity.

Eye Irritation Test (USP/NF)*

This test evaluates the potential for ocular irritation resulting from residual chemical leachables in contact lens materials. The effects are assessed *in vivo* using rabbits.

In Vitro Cytotoxicity Test (USP/NF)*

This test evaluates the potential for cytotoxicity resulting from residual chemical leachables in contact with lens materials. The effects are assessed *in vitro* using cytotoxicity

* United States Pharmacopeia (USP) 24/National Formulary (NF) 19, 2000 (or current update).

studies (e.g., tissue culture–agar overlay method or a suitable validated alternative).

Additional recommended testing. The following tests are not required if the applicant provides appropriate documentation demonstrating that either of the following criteria have been met:

- The recommended lens care regimen has been approved for use with the specific lens material group.
- The plastic lens carries no charge or the same electric charge as the preservative system used in the approved care regimen.

However, the following tests are required if

- A lens material is manufactured using a new monomer not previously used in a currently marketed hydrophilic or hydrophobic lens
- A UV-absorber is incorporated into the material, unless a scientific justification is provided to the contrary (e.g., use of a UV-absorber that has been previously cleared by the manufacturer for use in contact lenses of the same generic class, that is, hydrophilic or hydrophobic materials), and will be incorporated into the lens by a method that has been approved in a PMA or cleared in a substantial equivalence [section 513(I)(1)(A)] premarket notification [510(k)] for the manufacturer

Sensitization Tests

Preservative Uptake and Release Contact lens polymers may absorb or adsorb preservative materials that could possess irritating or sensitizing properties that are potentially irritating to some users. A quantitative analysis of preservative uptake per lens, the amount released, and the time course of release is conducted. Results obtained from these test data are used to predict the potential for a preservative-related toxicity, as well as the potential for inducing a sensitivity/allergic response associated with the lens group.

*Skin Sensitization (Guinea Pig Maximization Test)** This test grades or ranks chemical constituents on a scale of I through V as to their potential for inducing a sensitivity response in the guinea pig model. The grade or ranking is based on the number of animals sensitized, and the results are classified on an ascending scale from a weak (grade I) to an extreme sensitizing agent (grade V).

Three-Week Ocular Irritation Test in Rabbits

This *in vivo* test of the contact lenses in rabbits is used as a biocompatibility test as well as a toxicity test of the lens material. The test assesses the effects of the ocular

environment on the lens material, as well as the effects of the lens material on the ocular tissues.

CONTACT LENS CARE PRODUCTS (i.e., SOLUTIONS, TABLETS)

The following *in vitro* and *in vivo* tests for contact lens care products are recommended by the Premarket Notification [510(k)] Guidance Document for Contact Lens Care Products, which was last revised in May 1997 by the Center for Devices and Radiological Health, FDA.

***In Vitro* Cytotoxicity Test (USP/NF)**

This test evaluates the potential for toxicity of the residual chemicals leaching from the lens into the lens care products (i.e., solution(s)/solubilized tablets). In addition, this test may be used to detect potential toxic carryover from uptake/release of the solution by the lens. The tissue culture–agar diffusion test, direct contact test, and/or elution test, or suitable validated alternative method may be used.

Acute Ocular Irritation Test

This test evaluates the potential for ocular irritation resulting from residual chemical leachables from the finished device that may be extracted in the lens care products (i.e., solution(s)/solubilized tablets). This method is also used to detect the potential for ocular irritation due to carryover from uptake/release of the solution by the lens and from direct instillation of an in-eye solution. This test should not be needed in cases where formulations contain known ocular irritants. In such cases, an appropriate warning should be required on the label for products known to cause ocular irritation (i.e., daily cleaners/periodic cleaners) in lieu of performing the test.

Acute Oral Toxicity Study

This study assesses the potential of the contact lens care product (i.e., solution(s)/solubilized tablets) to produce a toxic response as a result of deliberate or accidental ingestion of the product by adults or children. These data are used to determine the need for additional warnings or precautions in the labeling of the product for the purpose of consumer protection. For rodent testing, the maximum volume of an aqueous solution generally should not exceed 2 mL/100 g of body weight. This single large dose is referred to as the maximum tolerable dose (MTD). Should signs of toxicity be demonstrated at the MTD, further testing consistent with accepted toxicological practices is recommended to complete a risk/benefit assessment of the product.

Additional recommended testing: The following tests are recommended if a manufacturer is using a new preservative or an active ingredient/chemical component not previously used in a currently marketed contact lens care product:

1. *Skin Sensitization (Guinea Pig Maximization Test):* described earlier.
2. *In Vivo Ocular Biocompatibility Test (ISO 9394-1998):* This ISO test method, entitled “Optics and optical instruments—Determination of biological

* Magnusson, B. and Kligman, A.M., The identification of contact allergens by animal assay. The Guinea Pig Maximization Test, *J. Invest. Dermatol.*, 52(3), 268–276, 1969.

compatibility of contact lens material—Testing of the contact lens system by ocular study with rabbit eyes,” should be acceptable in its entirety to address preclinical ocular biocompatibility of contact lens products.

PLASTIC OPHTHALMIC CONTAINERS

In the Premarket Notification [510(k)] Guidance Document for Contact Lens Care Products (revised May 1997), the Center for Devices and Radiological Health of the FDA recommends the following *in vitro* and *in vivo* tests that are consistent with the procedures listed in the USP 24/NF19, Containers for Ophthalmics—Plastics (Biological Test Procedures):

1. Systemic injection test
2. Acute ocular irritation test
3. *In vitro* cytotoxicity test

These tests (described previously) indirectly or directly assess the potential toxicity of constituent(s) that may leach from the container for a prolonged period of time.

OPHTHALMIC THERAPEUTIC FORMULATIONS

Nonclinical study protocols, based on guidelines set forth by Goldenthal²¹ and Hackett,²⁶ have been developed to assess acute and systemic toxicity of ophthalmic formulations. The toxicity data developed from the following preclinical study designs are used to establish an adequate safety profile and assess risk.

One Day Acute Topical Ocular Irritation Test

This test is used for formulation ingredients that have not been previously used by the topical ocular route and that have been placed in Category 1 by FDA ophthalmic panel(s), for example, a single-application diagnostic drug used for producing mydriasis or a single-application topical anesthetic for producing corneal anesthesia. The test is designed to determine the ocular toxicity potential in the event of accidental or intentional drug misuse:

- Dosing should be according to the anticipated clinical regimen. However, the dosing frequency may be adjusted or exaggerated to enhance the chance of observing toxicity for the purpose of predicting human risk. Typically, formulation ingredients are instilled at 0.03–0.05 mL every 30 min for 6 consecutive hours, using at least 6 eyes (rabbit).
- When possible, use of multiples of the active ingredients is essential.
- If available, use of a marketed product (control) is included for comparison.

Subchronic (1–3 Months) and Chronic (≥1 Year) Topical Ocular Irritation with Systemic Toxicological Evaluations

These tests are intended for drugs that require multiple dose therapy. Therefore, testing requirements are more extensive

for the development of an adequate safety profile. The extent of testing depends on the intended use of the drug. For example, drugs administered intermittently (up to several times/day for treatment periods of 2 weeks to 3 months) for external eye disease (i.e., anti-inflammatory and antimicrobial drugs) require less comprehensive testing than drugs intended for chronic administration (daily for years or for remaining lifetime) for diseases such as glaucoma.

- If the ingredients have not been evaluated by any other route, have not been given a safe and effective rating by the FDA, or there are no published toxicology data, then systemic toxicity is monitored by including hematology, clinical chemistry, urinalysis, and histopathology of tissues, including the eyes. If the ingredients have adequate published safety data or if an FDA panel has placed them into Category 1 by another route of administration, then these additional parameters may not be required.
- The dosing regimen should be similar to that which is used clinically.
- When possible, use of multiples of the active ingredients is essential.
- If available, use of a marketed product (control) is included for comparison.

CHEMICAL SUBSTANCES (LIQUIDS, SOLIDS, AEROSOLS, AND LIQUIDS UNDER PRESSURE)

IRLG Guidelines

Eye irritation testing guidelines were developed by the Interagency Regulatory Liaison Group (IRLG), five federal agencies (Consumer Product Safety Commission, Occupational Safety and Health Administration, FDA, Environmental Protection Agency, and Food Safety and Quality Service of the Department of Agriculture) (Fed. Reg. 1977, 1979). Standardized guidelines for eye irritation (Fed. Reg. 1981)²² are summarized in the following section.

General Considerations

1. *Good laboratory practices.* Studies should be conducted according to good laboratory practice regulations (21 CFR, Part 58).
2. *Test substance.* As far as is practical, composition of the test substance should be known and should include the names and quantities of all major components, known contaminants and impurities, and the percentages of unknown materials. A lot of substances should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.
3. *Animals.* Healthy animals, without eye defects or irritation and not subjected to any previous

experimental procedures, must be used. The test animal shall be characterized as to species, strain, sex, weight, and/or age. Each animal must be assigned an appropriate identification number. Recommendations in The National Academies Press publication entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.

4. *Documentation.* Color photographic documentation may be used to verify gross and microscopic findings.

Specific Considerations

1. *Test preparation.* Testing should be performed on young, adult, albino rabbits (male or female) weighing approximately 2–3 kg. Other species may also be tested for comparative purposes. For a valid eye irritation test, at least six rabbits must survive the test for each test substance. A trial test on three rabbits is suggested. If the substance produces corrosion, severe irritation, or no irritation, no further testing is necessary. However, if equivocal responses occur, testing in at least three additional animals should be performed. If the test substance is intended for use in or around the eye, testing on at least six animals should be performed.
2. *Test procedure.* Both eyes of each animal in the test groups must be examined by appropriate means within 24 h before substance administration. For most purposes, anesthetics should not be used; however, if the test substance is likely to cause more than momentary pain, local anesthetics may be used before instillation of the test substance for humane reasons. In such cases, anesthetics should be used only once, just before instillation of the test substance; the eye used as control in each rabbit should also be anesthetized. The test substance is placed in one eye of each animal by gently pulling the lower lid away from the globe (conjunctival culdesac) to form a cup into which the test substance is dropped. The lids are then gently held together for 1 s and the animal is released. The other eye, remaining untreated, serves as a control. Vehicle controls are not included unless it is suspected of causing irritation, in which case additional studies should be conducted using the vehicle as the test substance. For testing liquids, 0.1 mL is used. For solid, paste, or particulate substances (flake, granule, powder, or other particulate form), the amount used must have a volume of 0.1 mL, or a weight of not more than 100 mg. Substances comprising of bulky particulate or solids may be ground prior to administration. For aerosol products, the eye should be held open and the substance administered in a single, short burst for about 1 s at a distance of about 4 in. directly in front of the eye. The dose should be approximated by weighing the aerosol can before and after each

treatment for liquids. After the 24 h examination, the eyes may be washed, if desired. Tap water or isotonic saline solution of sodium chloride (USP or equivalent) should be used for all washings.

3. *Observations.* The eyes should be examined 24, 48, and 72 h after treatment. At the option of the investigator, the eyes may also be examined at 1 h and at 7, 14, and 21 days. In addition to the required observations of the cornea, iris, and conjunctivae, serious lesions such as pannus, phlyctena, and rupture of the globe should be reported. The grades of ocular reaction (see the "Grades for Ocular Lesions" section) must be recorded at each examination. Evaluation of reactions can be facilitated by using a binocular loupe, hand slit lamp, or other appropriate means. After the recording of observations at 24 h, the eyes of any or all rabbits may be examined further after applying fluorescein stain. An animal has exhibited a positive reaction if the test substance has produced one or more of the following signs at any observation:
 - a. Ulceration of the cornea (other than a fine stippling).
 - b. Inflammation of the iris (other than slight deepening of the rugae or light hyperemia of the circumcorneal blood vessels).
 - c. An obvious swelling in the conjunctivae (excluding the cornea and iris) with partial eversion of the eyelids or a diffuse crimson color with individual vessels not easily discernible.
4. *Evaluation.* The test result is considered positive if four or more animals in either test group exhibit a positive reaction. If only one animal exhibits a positive reaction, the test result is regarded as negative. If two or three animals exhibit a positive reaction, the investigator may designate the substance an irritant. When two or three animals exhibit a positive reaction and the investigator does not designate the substance an irritant, the test shall be repeated with a different group of six animals. The second test result is considered positive if three or more of the animals exhibit a positive reaction. Opacity grades 2–4 and/or perforation of the cornea are considered to be corrosive effects or when opacities persist for 21 days. If only one or two animals in the second test exhibit a positive reaction, the test should be repeated with a different group of six animals. When a third test is needed, the substance will be regarded as an irritant if any animal exhibits a positive response.

Data Reporting

1. *Identification.* Each test report should be signed by the persons responsible for the test, identify the laboratory where the test was performed by name and address, and give inclusive dates of the test.
2. *Body of report.* The test report must include all information necessary to provide a complete and

accurate description and evaluation of the test procedures and results in the following sections:

- a. Summary and conclusions.
- b. Materials, including the identification of the test substance (chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition), manufacturer and lot number of the substance tested, and specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance. Specific animal data are to be included in the report. This includes species and strain, source of supply of the animals, description of any pretest acclimation, and number, age, and condition of animals of each sex in each test group.
- c. Methods, such as deviation from guidelines, specifications of test methods, data on dosage administration, and data on observation methods.
- d. Results, such as tabulation of individual animal data must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show relation of effects to time of dosing, etc.

OECD Guidelines

The Organization for Economic Cooperation and Development (OECD) Guideline for "Acute Eye Irritation/Corrosion," No. 405 (Adopted April 24, 2002).²³

Introductory Information

Prerequisites

- Solid or liquid test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- pH and buffer capacity (where appropriate)
- Melting point/boiling point
- Analyses of extant data, or development of relevant data through sequential testing
- Performance of a validated and accepted *in vitro* eye irritation test
- Performance of an OECD Guideline study "Dermal Irritation/Corrosion," No. 404 (Adopted April 24, 2002)

Standard Document There are no relevant international standards.

Method

Introduction, Purpose, Scope, Relevance Application, and Limits of Test In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on eyes of mammals is an important initial step. Information derived from this test serves to

indicate the possible existence of hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

Definitions

Eye irritation is the production of reversible changes in the eye after the application of a test substance to the anterior surface of the eye.

Eye corrosion is the production of irreversible tissue damage in the eye after application of a test substance to the anterior surface of the eye.

Principle of the Test Method The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation/corrosion is evaluated and scored at specific intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed.

Initial Considerations All the available information on a substance must be considered carefully to minimize the testing of substances under conditions that are likely to produce severe reactions. The following information may be useful in this regard.

1. **Physicochemical properties and chemical reactivity.** Strongly acidic or alkaline substances, for example, which can be expected to result in a pH in the eye of 2 or less, or 11.5 or greater, need not be tested because of their probable corrosive properties. Buffer capacity also should be considered.
2. **Results from skin irritation studies.** Materials that have demonstrated definite corrosive or severe skin irritancy in a dermal study need not be tested further for eye irritancy, presuming that such substances will produce similar severe effects on the eyes.
3. **Results from well-validated alternative studies.** Materials that have demonstrated potential corrosive or severe irritancy need not be tested further for eye irritation, presuming that such substances will produce similar severe effects on the eyes in a test using this guideline.

Description of the Test Procedure

Preparations Both eyes of each experimental animal provisionally selected for testing should be examined within 24 h before testing starts. Animals showing eye irritation, ocular defects, or preexisting corneal injury should not be used.

Experimental Animals

Selection of species. A variety of experimental animals have been used, but it is recommended that testing should be performed using healthy adult albino rabbits.

Single animal test. A single-animal test should be considered if marked effects are anticipated. If the results of this test in one rabbit suggest that the substance is severely irritant (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further testing for ocular irritancy in subsequent animals may not need to be conducted. Occasionally, further testing in additional animals may be appropriate to investigate specific aspects.

Number of animals. In cases other than a single animal test, at least three animals should be used. Additional animals may be required to clarify equivocal responses.

Housing and feeding conditions. Animals should be housed individually. The room temperatures for experimental animals should be 22°C ($\pm 3^\circ\text{C}$) for rodents and 20°C ($\pm 3^\circ\text{C}$) for rabbits; the relative humidity should be 30%–70%. Where the lighting is artificial, the sequence should be 12 h light/12 h dark. Conventional laboratory diets are suitable for feeding, and an unrestricted supply of drinking water should be available.

Test Conditions Dose Level

1. *Testing of solids and liquids.* For testing liquids, a dose of 0.1 mL is used. Pump sprays should not be used, but the liquid should be expelled instead, and 0.1 mL collected and instilled in the eye as described for liquids. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0.1 mL, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular, it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them, for example, by tapping the measuring container.

Testing of aerosols. To test a substance contained in a pressurized aerosol container, the eye should be held open and the test substance administered in a single burst of about 1 s from a distance of 10 cm directly in front of the eye. Care should be taken not to damage the eye. In appropriate cases, aerosols may be tested in the manner already described for pump sprays.

An estimate of the dose may be made by simulating the test as follows: the substance is sprayed through a window the size of a rabbit eye, placed directly before a weighing paper. The weight increase of the weighing paper is considered to approximate the amount sprayed into a rabbit eye. For volatile substances, the dose may be estimated by weighing the container before and after use.

2. *Observation period.* The duration of the observation period should not be fixed rigidly but should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. It usually should not exceed 21 days after instillation.

Procedure

Application The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about 1 s to prevent loss of the material. The other eye, which remains untreated, serves as a control.

Local Anesthetics If it is thought that the substance might cause more than momentary pain, a local anesthetic may be used before instillation of the test substance. The type and concentration of the local anesthetic should be selected carefully to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anesthetized.

Irritation The eyes of the test animals should not be washed out for 24 h after instillation of the test substance. At 24 h, a washout may be used if considered appropriate.

For some substances shown to be irritating by this test, additional tests using rabbits with eyes washed soon after instillation of the substance may be indicated. In these cases, it is recommended that three rabbits be used. Half a minute after instillation, the eyes of the rabbits are washed for half a minute using a volume and velocity of flow that will not cause injury.

Clinical Observations and Scoring The eyes should be examined at 1, 24, 48, and 72 h. If there is no evidence of irritation at 72 h the study may be ended. Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the conjunctivae, cornea, iris, and any other lesions that are noted should be recorded and reported. The grades of ocular reaction (see the “Grades for Ocular Lesions” section) should be recorded at each examination.

Examination of reactions can be facilitated by use of a binocular loupe, hand slit lamp, biomicroscope, or other suitable devices. After recording the observations at 24 h, the eyes of any or all rabbits may be examined further using fluorescein.

The grading of ocular responses is subject to various interpretations. To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used.

Data and Reporting

Treatment of Results Data may be summarized in tabular form, showing for each individual animal the irritation scores at the designated observation time, a description of the degree and nature of irritation, the presence of serious lesions, and any effects other than ocular that were observed.

Evaluation of the Results The ocular irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant

properties of a material. They should be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

Test Reports The test report should include the following information:

- Species/strain used
- Physical nature and, where applicable, concentration and pH value of the test substance
- Tabulation of irritant/corrosive response data for each animal at each observation time (e.g., 1, 24, 48, and 72 h)
- Description of any serious lesions observed
- Narrative describing the degree and nature of irritation or corrosion observed
- Description of the method used to score the irritation at 1, 24, 48, and 72 h (e.g., hand slit lamp, bio-microscope, fluorescein)
- Description of any nonocular topical effects noted

Interpretation of the Results Extrapolation of the results of eye irritation studies in animals to man is valid only to a limited degree. The albino rabbit is more sensitive than man to ocular irritants or corrosives in most cases. Similar results in tests on other animal species can give more weight to extrapolation from animal studies to man.

Care should be taken in the interpretation of data to exclude irritation resulting from secondary infection.

OCSPP 870.2400 Acute Eye Irritation

The Office of Chemical Safety and Pollution Prevention (OCSPP) developed this guideline through a process of harmonization that blended the testing guidance and requirements of the OCSPP, the Office of Pesticide Programs (OPP), and the Organization for Economic Cooperation and Development (OECD).

Scope

1. **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 USC 136, et seq.) and the US Environmental Protection Agency under the Toxic Substances Control Act (15 USC 2601).
2. **Background.** The source materials used in developing this harmonized OCSPP test guideline are EPA OTS 798.4500 Primary Eye Irritation; OPP 81-4 Acute Eye Irritation—Rabbit (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals); EPA report 540/09-82-025, 1982; and OECD 405 Acute Eye Irritation/Corrosion.

Purpose

1. In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosion effects on eyes of mammals is

an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

2. Data on primary eye irritation are required by 40 CFR 158.340 to support the registration of each manufacturing-use product and end-use product. (See §158.50 to determine whether these data must be submitted and which purity/grade of the test substance should be tested.)

Definitions

The definitions in Section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice (GLP) standards apply to this test guideline. The following definitions also apply to this guideline.

Eye corrosion is the production of irreversible tissue damage in the eye following application of a test substance to the anterior surface of the eye.

Eye irritation is the production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye.

Principle of the Test Method

The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation/corrosion is evaluated and scored at specified intervals and is fully described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed. The period of observation should be at least 72 h, but need not exceed 21 days. Animals showing severe and enduring signs of distress and pain may need to be killed in a humane fashion.

Initial Considerations

1. Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested owing to their predictable corrosive properties. Buffer capacity should be taken into account.
2. Materials that have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation. It may be presumed that such substances will produce similar severe effects in the eyes.
3. Results from well-validated and accepted *in vitro* test systems may serve to identify corrosives or irritants such that the test material need not be tested *in vivo*.

Test Procedures

1. **Animal selection**
 - a. **Species and strain.** A variety of experimental animals have been used, but it is recommended that testing should be performed using healthy

adult albino rabbits. Commonly used laboratory strains should be used. If any other mammalian species is used, the tester should provide justification/reasoning for its selection.

- b. *Number of animals.* A single animal should be considered if marked effects are anticipated. If the results of this test in one animal suggest the test substance to be a severe irritant (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further tests may not need to be performed. In cases other than a single animal test, at least three animals should be used. Occasionally, further testing in additional animals may be appropriate to clarify equivocal responses.
2. *Dose level.* For testing liquids, a dose of 0.1 mL is recommended. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0.1 mL, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular, it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them (e.g., by tapping the measuring container). To test a substance contained in a pressurized aerosol container, the eye should be held open and the test substance administered in a single burst of about 1 s duration from a distance of 10 cm directly in front of the eye. The dose may be estimated by weighing the container before and after use. Care should be taken not to damage the eye. Pump sprays should not be used, but instead the liquid should be expelled and 0.1 mL collected and instilled into the eye as described for liquids. For volatile substances, the dose may be estimated by weighing the container before and after use.
3. *Examination of eyes prior to test.* Both eyes of each experimental animal provisionally selected for testing should be examined within 24 h before testing starts by the same procedure to be used during the test examination. Animals showing eye irritation, ocular defects, or preexisting corneal injury should not be used.
4. *Application of test substance*
 - a. The test substance should be placed in the conjunctival sac of the eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about 1 s to limit the loss of the material. The other eye, which remains untreated, serves as a control. If it is thought that the substance may cause extreme pain, local anesthetic may be used prior to instillation of the test substance. The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anesthetized.
 - b. The eyes of the test animals should not be washed out for 24 h following instillation of the test substance. At 24 h, a washout may be used if considered appropriate. This is to show whether washing with water palliates or exacerbates irritation.
 - c. For some substances shown to be irritating by this test, additional testing using animals with eyes washed soon after instillation of the substance may be indicated. Half a minute after instillation, the eyes of the animals are washed with water for 30 s, using a volume and velocity of flow that will not cause injury.
5. *Observation period.* The duration of the observation period is at least 72 h, and should not be fixed rigidly, but should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. The observation period normally need not exceed 21 days after instillation.
6. *Clinical examination and scoring*
 - a. The eyes should be examined at 1, 24, 48, and 72 h. If there is no evidence of irritation at 72 h, the study may be ended. Extended observation (e.g., at 7 and 21 days) may be necessary if there is persistent corneal involvement or other ocular irritation to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the cornea, iris, and conjunctivae, any other lesions that are noted should be recorded and reported. The grades for ocular reactions (using the grading system in the "Grades for Ocular Lesions" section) should be recorded at each examination.
 - b. Examination of reactions can be facilitated by use of a binocular loupe, hand slit lamp, biomicroscope, or other suitable device. After recording the observations at 24 h, the eyes of any or all rabbits may be further examined with the aid of fluorescein.
 - c. The grading of ocular responses is subject to various interpretations. To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used.

Data and Reporting

1. *Data summary.* Data should be summarized in tabular form, showing for each individual animal the irritation scores at observation time until resolution occurs (nonpositive grades) or a maximum of 21 days, when the test is concluded; a description of the degree and nature of irritation, the presence of serious lesions, and any effects other than ocular that were observed should be provided.
2. *Evaluation of the results.* The ocular irritation scores should be evaluated in conjunction with

the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

3. *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

- a. Species, strain, sex, age, and source of test animal
- b. Rationale for selection of species (if the species is other than the species preferred)
- c. Tabulation of irritant/corrosive response data for each individual animal at each observation time point (e.g., 1, 24, 48, and 72 h until reversibility of lesions or termination of the test)
- d. Description of any lesions observed
- e. Narrative description of the degree and nature of irritation or corrosion observed
- f. Description of the method used to score the irritation at 1, 24, 48, and 72 h (e.g., hand slit lamp, biomicroscope, fluorescein stain)
- g. Description of any nonocular effects noted
- h. Description of any pretest conditioning, including diet, quarantine, and treatment of disease
- i. Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animals
- j. Manufacture, source, purity, and lot number of test substance
- k. Physical nature and, where appropriate, concentration and pH value of the test substance
- l. Identification, composition, and characteristics of any vehicles (e.g., diluents, suspending agents, emulsifiers, and anesthetics) or other materials used in administering the test substance
- m. A list of references cited in the body of the report, that is, references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results

OCULAR SCORING CRITERIA

SCALE OF WEIGHTED SCORES FOR GRADING THE SEVERITY OF OCULAR LESIONS DEVELOPED BY DRAIZE ET AL.

In 1944, Draize et al.²⁴ described an eye irritancy grading system for evaluating drugs and other materials intended for use in or around the eye. Numerical scores were assigned for reactions of cornea, iris, and conjunctivae. The total ocular irritation score was calculated by a formula that gave the greatest weight to corneal changes (total

maximum = 80). A total maximum score = 10 for the iris, and 20 for the conjunctiva.

1. Cornea

- A. Opacity Degree of Density (area which is most dense is taken for reading)

Scattered or diffuse area—details of iris clearly visible	1
Easily discernible translucent areas—details of iris clearly visible	2
Opalescent areas—no details of iris visible; size of pupil barely discernible	3
Opaque—iris invisible	4
 - B. Area of Cornea Involved

One quarter (or less) but not zero	1
Greater than one quarter—less than one half	2
Greater than one half—less than three quarters	3
Greater than three quarters—up to whole area	4
- Score equals $A \times B \times 5$
Total maximum = 80

2. Iris

- A. Values

Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage; gross destruction (any one or all of these)	2
- Score equals $A \times 5$
Total possible maximum = 10

3. Conjunctivae

- A. Redness (refers to palpebral conjunctivae only)

Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
 - B. Chemosis

Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
 - C. Discharge

Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
- Score $(A + B + C) \times 2$
Total maximum = 20

Note: The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae.

GRADES FOR OCULAR LESIONS

The following standardized grading system is used in testing guidelines of several US federal agencies (Consumer Product Safety Commission, Occupational Safety and Health Administration, FDA, Environmental Protection Agency, and Food Safety and Quality Service of the Department of Agriculture) and the Organization for Economic Cooperation and Development (OECD) member countries.

Cornea

Opacity: degree of density (area most dense taken for reading)

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster, details of iris clearly visible)	1 ^a
Easily discernible translucent areas, details of iris slightly obscured	2
Nacreous areas, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4

Iris

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperemia, or injection—any of these or any combination thereof, iris still reacting to light (sluggish reaction is positive)	1 ^a

No reaction to light, hemorrhage, gross destruction (any or all of these) 2

Conjunctivae

Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)

Blood vessels normal	0
Some blood vessels definitely hyperemic (injected)	1
Diffuse, crimson color, individual vessels not easily discernible	2 ^a
Diffuse beefy red	3
Chemosis: lids and/or nictitating membranes	
No swelling	0
Any swelling above normal (includes nictitating membranes)	1
Obvious swelling with partial eversion of lids	2 ^a
Swelling with lids about half closed	3
Swelling with lids more than half closed	4

^a Readings at these numerical values or greater indicate positive responses.

REPRESENTATIVE ILLUSTRATIONS OF DRAIZE

EYE IRRITATION SCORES

Figures 4.6 through 4.11 are intended to illustrate the subjective grades for corneal, conjunctival, and iridial manifestations of ocular irritation. Each plate is reproduced directly from the *Illustrated Guide for Grading Eye Irritation Caused by Hazardous Substances*, Consumer Product Safety Commission, Washington, DC, 1972.

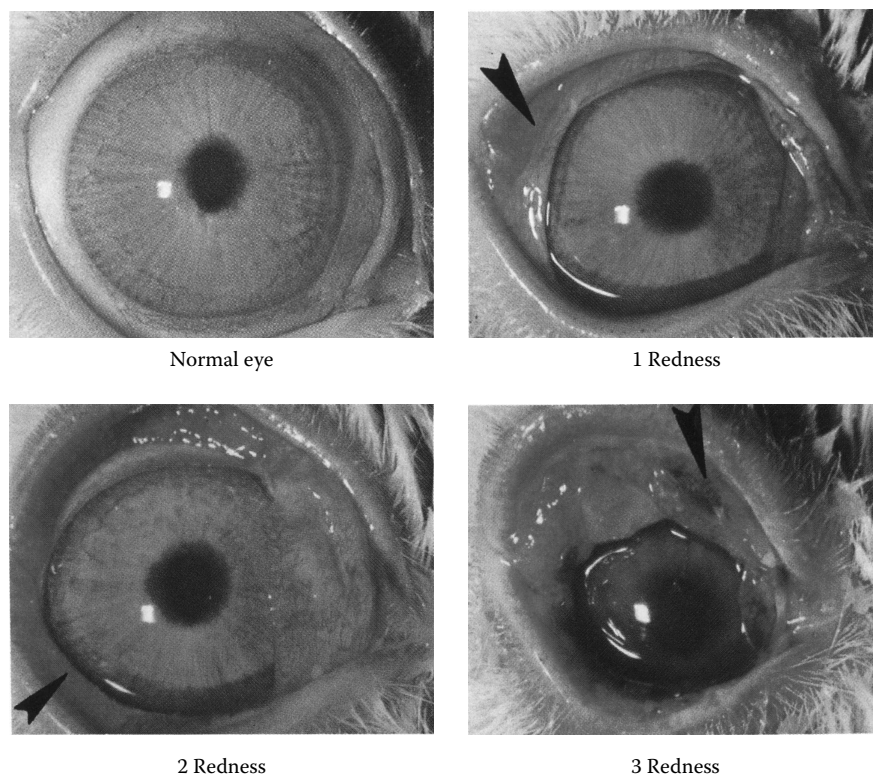


FIGURE 4.6 (See color insert.) Photographs demonstrating the four grades for conjunctival redness. Conjunctival redness is typically not homogeneous; therefore, only the most severely affected area of the conjunctiva should be graded as shown by the arrows in the photographs.

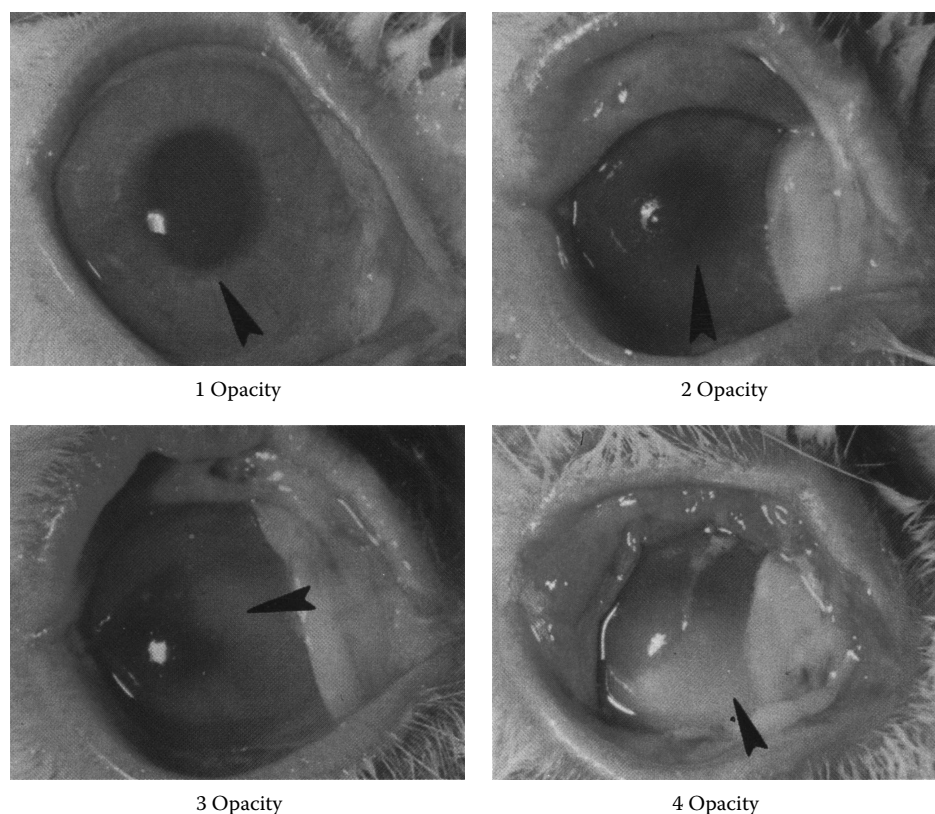


FIGURE 4.7 (See color insert.) Photographs demonstrating the four grades for corneal opacity. Because a corneal lesion is not distributed homogeneously, the most severely affected part of the cornea (see arrows) is graded.

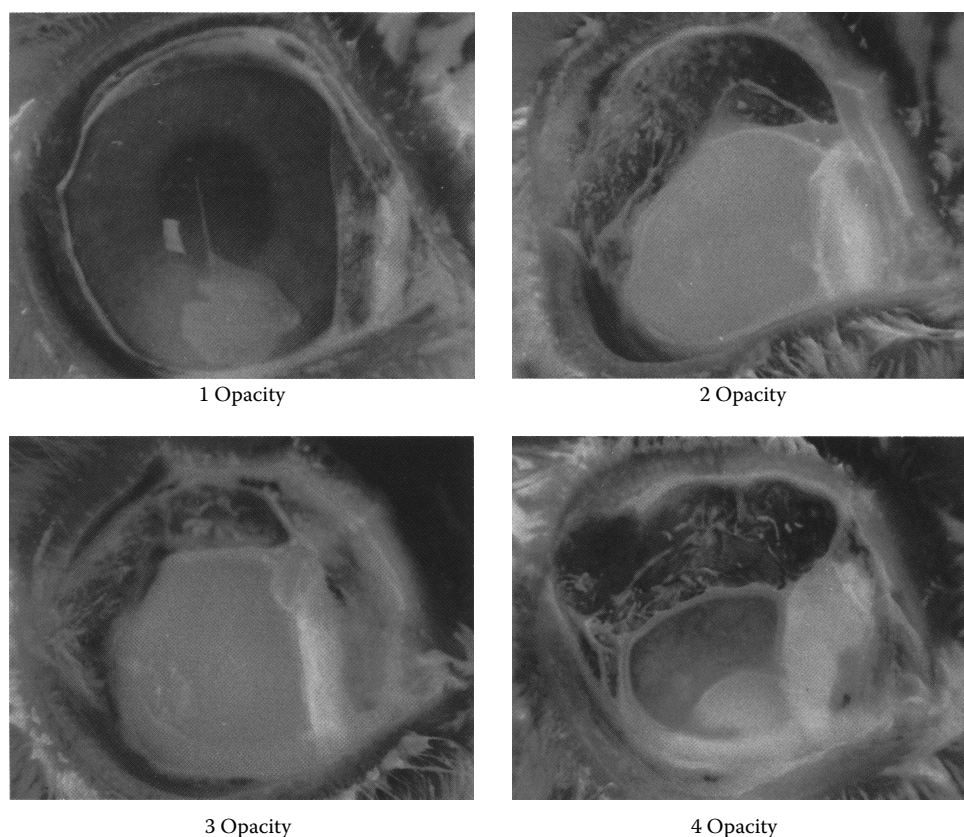


FIGURE 4.8 (See color insert.) Eyes stained with fluorescein and photographed under UV light. Fluorescein-stained areas demarcate corneal erosion, not opacity. Therefore, the areas stained with fluorescein do not necessarily correspond to the grades for opacity.

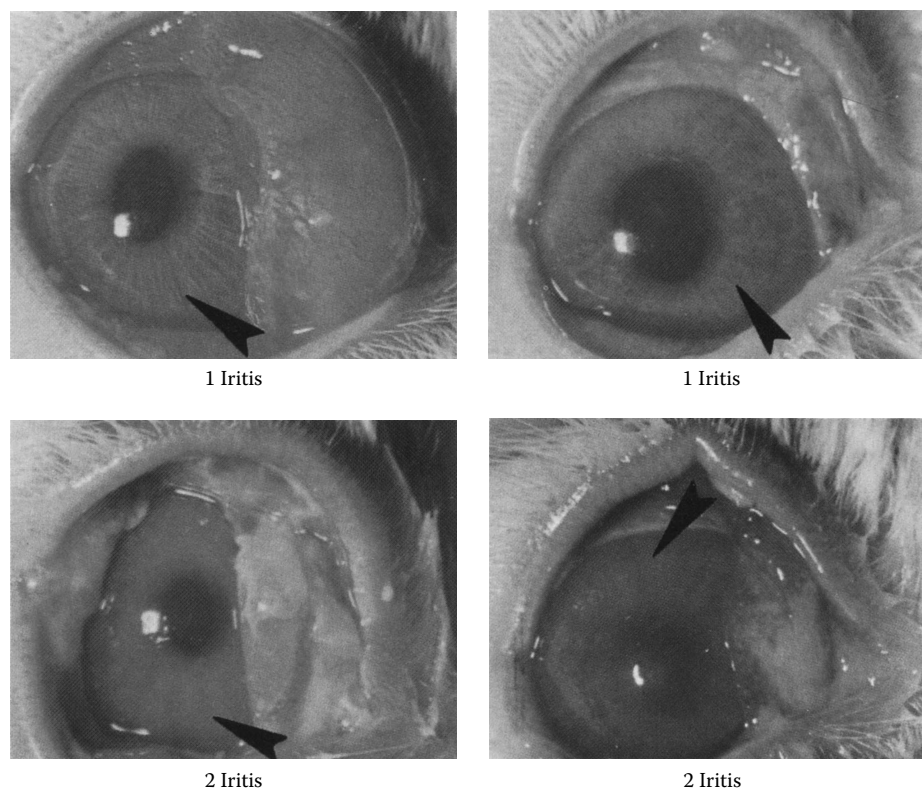


FIGURE 4.9 (See color insert.) Two grades for iritis demonstrated. Grade 1 is a deepening of iridial rugae, or injection (hyperemia) of iridial vessels. The upper photograph clearly shows injection of the secondary vessels of the iris, but this finding is more difficult to perceive in the upper right photograph due to loss of corneal clarity. Grade 2 iritis involves no reaction to light, hemorrhage, and/or destruction of the iris. Since this is almost invariably accompanied by significant corneal opacity, it may be difficult to observe. The two lower photographs demonstrate hemorrhage of the iris.

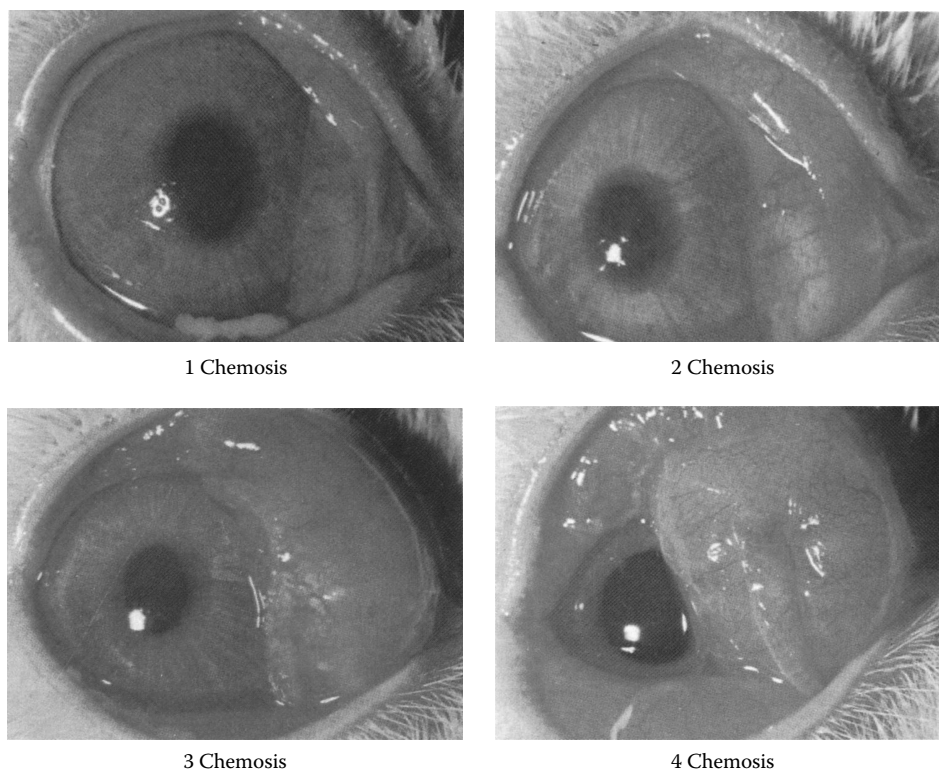


FIGURE 4.10 (See color insert.) Photographs intended to indicate the degree of difference between each conjunctival grade. They may not accurately represent chemosis because the eyes have been held open to show other aspects of irritation.

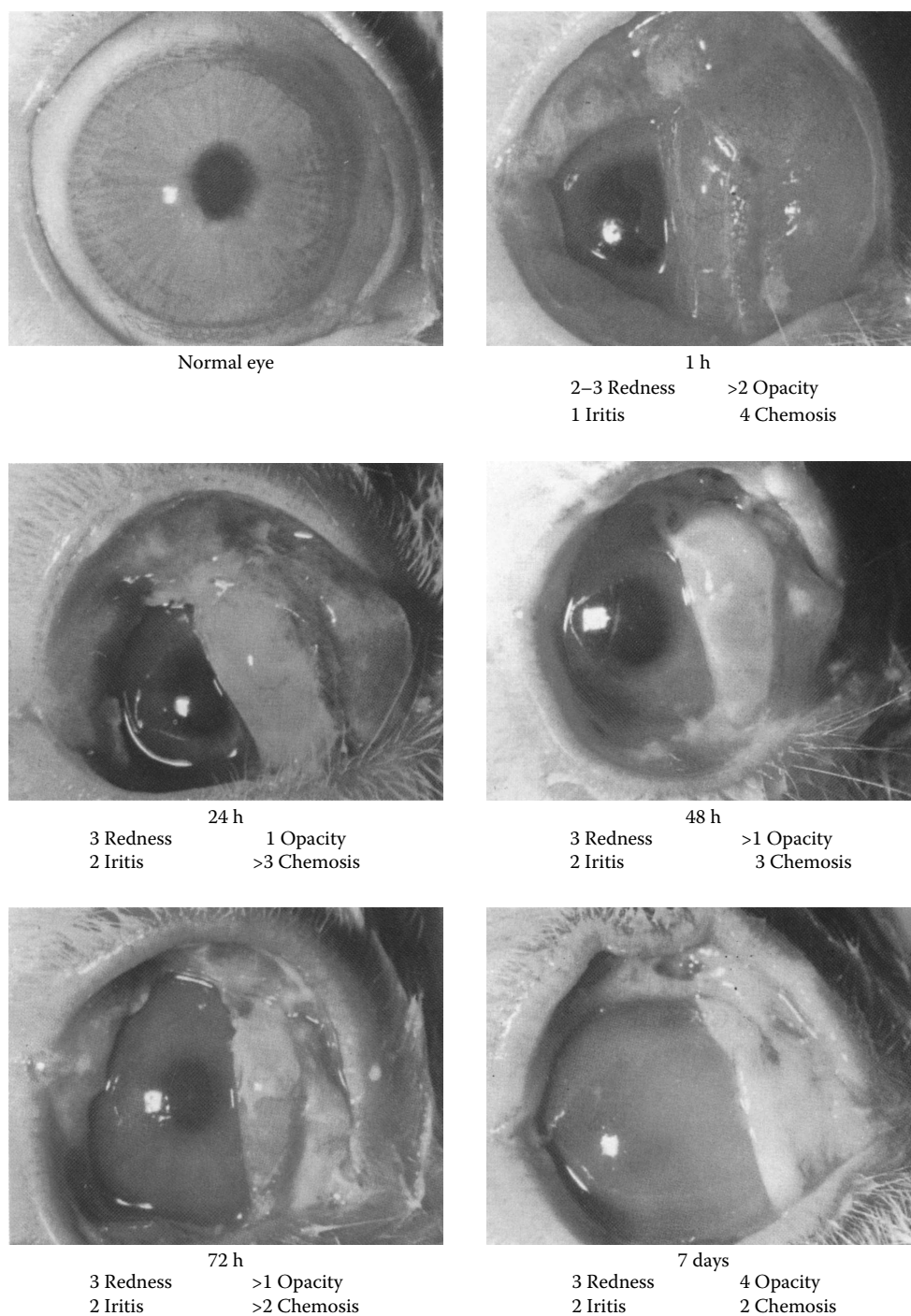


FIGURE 4.11 (See color insert.) A time sequence of the same eye shown from before administration of an irritant to 7 days after exposure.

SCORING CRITERIA FOR OCULAR EFFECTS OBSERVED IN SLIT LAMP MICROSCOPY

Location of Observations	Grades
<i>Corneal observations</i>	
Intensity	
Only epithelial edema (with only slight stromal edema or without stromal edema)	1
Corneal thickness 1.5 × normal	2
Corneal thickness 2 × normal	3
Cornea entirely opaque so that corneal thickness cannot be determined	4
Area involved	
≤25% of total corneal surface	1
>25% but ≤50%	2
>50% but ≤75%	3
>75%	4
Fluorescein staining	
≤25% of total corneal surface	1
>25% but ≤50%	2
>50% but ≤75%	3
>75%	4
Neovascularization and pigment migration	
≤25% of total corneal surface	1
>25% but ≤50%	2
>50% but ≤75%	3
>75%	4
Perforation	4
Maximal corneal score	20
<i>Iridal observations</i>	
Iritis is quantitated by the cells and flare in the anterior chamber, iris, hyperemia, and capillary light reflex	
Cells in aqueous chamber	
A few	1
A moderate number	2
Many	3
Aqueous flare (Tyndall effect)	
Slight	1
Moderate	2
Marked	3
Iris hyperemia	
Slight	1
Moderate	2
Marked	3
Pupillary reflex	
Sluggish	1
Absent	2
Maximal iridal score	11
<i>Conjunctival observations</i>	
Hyperemia	
Slight	1
Moderate	2
Marked	3
Chemosis	
Slight	1
Moderate	2
Marked	3

(continued)

Location of Observations	Grades
Fluorescein staining	
Slight	1
Moderate	2
Marked	3
Ulceration	
Slight	1
Moderate	2
Marked	3
Scarring	
Slight	1
Moderate	2
Marked	3
Maximal conjunctival score	15

Source: Chan, P-K. and Hayes, A.W., in *Toxicology of the Eye, Ear, and Other Special Senses*, Hayes, A.W., Ed., Raven Press, New York, 1985. With permission.

OCULAR SCORING SYSTEM FOR RABBITS BASED ON SLIT LAMP EXAMINATION*

Conjunctiva

Conjunctival changes can be divided clinically into congestion, swelling (chemosis), and discharge. Generally, the sequence of events for these changes is congestion, discharge, and swelling.

Conjunctival Congestion

0 = Normal. May seem blanched to reddish pink without perilimbal injection (except at 12 and 6 o'clock positions) with vessels of the palpebral and bulbar conjunctivae easily observed.

+1 = A flushed, reddish color predominantly confined to the palpebral conjunctiva with some perilimbal injection but primarily confined to the lower and upper parts of the eye from the 4, 7, 11, and 1 o'clock positions.

+2 = Bright red color of the palpebral conjunctiva with accompanying perilimbal injection covering at least 75% of the circumference of the perilimbal region.

+3 = Dark, beefy red color with congestion of both the bulbar and the palpebral conjunctivae along with pronounced perilimbal injection and the presence of petechia on the conjunctiva. The petechiae generally predominate along the nictitating membrane and the upper palpebral conjunctiva.

Conjunctival Swelling

There are five divisions from 0 to +4.

0 = Normal or no swelling of the conjunctival tissue.

+1 = Swelling above normal without eversion of the lids (can be ascertained easily by noting that the upper

* Reproduced from Marzulli, F.N. and Maibach, H.I., Eds., *Dermatotoxicology*, 4th edn., Hemisphere, New York, 1991, pp. 780–785. With permission.²⁶

and lower eyelids are positioned as in the normal eye); swelling generally starts in the lower culdesac near the inner canthus, which requires slit lamp examination.

- +2 = Swelling with misalignment of the normal approximation of the lower and upper eyelids; primarily confined to the upper eyelid so that in the initial stages the misapproximation of the eyelids begins by partial eversion of the upper eyelid. In this stage, swelling is confined generally to the upper eyelid, although it exists in the lower culdesac (observed best with the slit lamp).
- +3 = Swelling definite with partial eversion of the upper and lower eyelids essentially equivalent. This can be easily ascertained by looking at the animal head-on and noticing the positioning of the eyelids; if the eye margins do not meet, eversion has occurred.
- +4 = Eversion of the upper eyelid is pronounced with less pronounced eversion of the lower eyelid. It is difficult to retract the lids and observe the perilimbal region.

Conjunctival Discharge

Discharge is defined as a whitish gray precipitate, which should not be confused with the small amount of clear, inspissated, mucoid material that can be formed in the medial canthus of a substantial number of rabbit eyes. This material can be removed with a cotton swab before the animals are used.

- 0 = Normal. No discharge.
- +1 = Discharge above normal and present on the inner portion of the eye but not on the lids or hairs of the eyelids. The small amount that is in the inner and outer canthus can be ignored if it has not been removed before starting the study.
- +2 = Discharge is abundant, easily observed, and has collected on the lids and around the hairs of the eyelids.
- +3 = Discharge has been flowing over the eyelids, wetting the hairs substantially on the skin around the eye.

Aqueous Flare

The intensity of the Tyndall phenomenon is scored by comparing the normal Tyndall effect observed when the slit lamp beam passes through the lens with that seen in the anterior chamber. The presence of aqueous flare is the presumptive evidence of breakdown of the blood–aqueous barrier.

- 0 = Absence of visible light beam in the anterior chamber (no Tyndall effect).
- +1 = The Tyndall effect is barely discernible. The intensity of the light beam in the anterior chamber is less than the intensity of the slit beam as it passes through the lens.
- +2 = The Tyndall beam in the anterior chamber is easily discernible and is equal in intensity to the slit beam as it passes through the lens.
- +3 = The Tyndall beam in the anterior chamber is easily discernible; its intensity is greater than the intensity of the slit beam as it passes through the lens.

Light Reflex

The pupillary diameter of the iris is controlled by the radial and sphincter muscles. Contraction of the radial muscle due to adrenergic stimulation results in mydriasis, whereas contraction of the sphincter muscle due to cholinergic stimulation results in miosis. Because an ophthalmic drug can exert potential effects on these neural pathways, it is important to assess the light reflex of an animal as part of the ophthalmic examination. Using full illumination with the slit lamp, the following scale is used:

- 0 = Normal pupillary response
- 1 = Sluggish pupillary response
- 2 = Maximally impaired (i.e., fixed) pupillary response

Iris Involvement

In the following definitions, the primary, secondary, and tertiary vessels are used as an aid to determine a subjective ocular score for iris involvement. The assumption is made that the greater the hyperemia of the vessels and the more the secondary and tertiary vessels are involved, the greater the intensity of iris involvement. The scores range from 0 to +4.

- 0 = Normal iris without any hyperemia of the iris vessels. Occasionally around the 12 to 1 o'clock position near the pupillary border and around the 6 and 7 o'clock position near the pupillary border there is a small area about 1–3 mm in diameter in which both the secondary and tertiary vessels are slightly hyperemic.
- +1 = Minimal injection of secondary vessels but not tertiary. Generally, it is uniform, but may be of greater intensity at the 1 or 6 o'clock position. If it is confined to the 1 or 6 o'clock position, the tertiary vessels must be substantially hyperemic.
- +2 = Minimal injection of tertiary vessels and minimal to moderate injection of the secondary vessels.
- +3 = Moderate injection of the secondary and tertiary vessels with slight swelling of the iris stroma. This gives the iris surface a slightly rugose appearance, which is usually most prominent near the 3 and 9 o'clock positions.
- +4 = Marked injection of the secondary and tertiary vessels with marked swelling of the iris stroma. The iris seems rugose; may be accompanied by hemorrhage (hyphema) in the anterior chamber.

Cornea

The scoring scheme measures the severity of corneal opacity and the area of the cornea involved. The severity of corneal opacity is graded as follows:

- 0 = Normal cornea. Appears, with the slit lamp adjusted to a narrow slit, as having a bright gray line on the epithelial surface and a bright gray line on the endothelial surface with a marble-like gray appearance of the stroma.
- +1 = Some loss of transparency. Only the anterior half of the stroma is involved as observed with an optical section of the slit lamp. The underlying

structures are clearly visible with diffuse illumination, although some cloudiness can be readily apparent with diffuse illumination.

- +2 = Moderate loss of transparency. In addition to involving the anterior stroma, the cloudiness extends all the way to the endothelium. The stroma has lost its marble-like appearance and is homogeneously white. With diffuse illumination, underlying structures are clearly visible.
- +3 = Involvement of the entire thickness of the stroma. With optical section, the endothelial surface is still visible. However, with diffuse illumination the underlying structures are just barely visible (to the extent that the observer is still able to grade flare and iritis, observe for pupillary response, and note lenticular changes).
- +4 = Involvement of the entire thickness of the stroma. With the optical section, cannot clearly visualize the endothelium. With diffuse illumination, the underlying structures cannot be seen. Cloudiness removes the capability for judging and grading flare, iritis, lenticular changes, and pupillary response.

The surface area of the cornea relative to the area of opacity is divided into five grades from 0 to +4.

- 0 = Normal cornea with no area of opacity
- +1 = 1%–25% area of stromal opacity
- +2 = 25%–50% area of stromal opacity
- +3 = 51%–75% area of stromal opacity
- +4 = 76%–100% area of stromal opacity

Pannus is vascularization or the penetration of new blood vessels derived from the limbal vascular loops into the corneal stroma. Pannus is divided into three grades.

- 0 = No pannus.
- +1 = Vascularization is present but vessels have not invaded the entire corneal circumference. Where localized vessel invasion has occurred, vessels have not penetrated beyond 2 mm.
- +2 = Vessels have invaded 2 mm or more around the entire corneal circumference.

The use of fluorescein is a valuable aid in defining epithelial damage. For fluorescein staining, the area can be scored on a 0 to +4 scale.

- 0 = Absence of fluorescein staining.
- +1 = Slight fluorescein staining confined to a small focus. With diffuse illumination, the underlying structures are easily visible. (The outline of the pupillary margin is as if there were no fluorescein staining.)
- +2 = Moderate fluorescein staining confined to a small focus. With diffuse illumination, the underlying structures are clearly visible, although there is some loss of detail.
- +3 = Marked fluorescein staining. Staining may involve a larger portion of the cornea. With diffuse

illumination, underlying structures are barely visible but are not completely obliterated.

- +4 = Extreme fluorescein staining. With diffuse illumination, the underlying structures cannot be observed.

Interpretation is facilitated by rinsing the eye with an isotonic irrigating solution to remove excess and nonabsorbed fluorescein.

Slit lamps are equipped with cobalt blue filters, which can be placed in front of the light from the slit illuminator to excite fluorescence of the fluorescein. Photographs using fluorescein staining require the use of this filter, and fluorescence will be enhanced by a yellow filter placed in front of the objectives of the corneal microscope.

Lens

The crystalline lens is readily observed with the aid of the slit lamp biomicroscope, and the location of lenticular opacity can be discerned readily by direct and retroillumination. The location of lenticular opacities can be divided arbitrarily into the following lenticular regions beginning from the anterior: anterior capsular, anterior subcapsular, anterior cortical, posterior cortical, posterior subcapsular, and posterior capsular. The lens should be evaluated routinely during ocular evaluations and graded as either N (normal) or A (abnormal). The presence of lenticular opacities should be described and the location noted as defined earlier.

CLASSIFICATION SCHEMES

CLASSIFICATION OF COMPOUNDS BASED ON EYE IRRITATION PROPERTIES

This classification scheme developed by Kay and Calandra²⁷ utilizes the Draize scoring system to rate the irritating potential of substances.

Step 1

Using the Draize eye irritation scoring system, find the maximum mean total score for all three tissues (cornea, iris, and conjunctivae) occurring within the first 96 h after instillation for which the incidence of this score plus or minus 5 points is at least 40%.

Step 2

Choose an initial or “tentative rating” on the basis of the score found in Step 1 as follows:

Score from Step 1	Tentative Eye Irritation Rating	Symbol
0.0–0.5 points	Nonirritating	N
0.5–2.5 points	Practically nonirritating	PN
2.5–15 points	Minimally irritating	M ₁
15–25 points	Mildly irritating	M ₂
25–50 points	Moderately irritating	M ₃
50–80 points	Severely irritating	S
80–100 points	Extremely irritating	E
100–110 points	Maximally irritating	M _x

For borderline scores, choose the higher rating

Step 3

Tentative Rating	Requirement for Maintenance
N	$MTS_{24} = 0$; for $MTS_{24} > 0$, raise one level
PN	As for N
M_1	$MTS_{48} = 0$; for $MTS_{48} > 0$, raise one level
M_2	$MTS_{96} = 0$; for $MTS_{96} > 0$, raise one level
M_3	1. $MTS_f \leq 20$; for $MTS_f > 20$, raise one level 2. $ITS_f = 10$ (60%); if not true then no rabbit may show $ITS_f \geq 30$; otherwise raise one level
S	1. As for M_3 , except use $MTS_f \leq 40$ 2. As for M_3 , except use $ITS_f \leq 30$ (60%) and 60 for high
E	1. As for M_3 , except use $MTS_f \leq 80$ 2. As for M_3 , except use $ITS_f \leq 60$ (60%) and 100 for high
M_x	1. $MTS_f > 80$ (60%); for $MTS_f \leq 80$, lower one level 2. $ITS_f > 60$ (60%); otherwise lower one level

Symbols: MTS, mean total score; ITS, individual rabbit total score; Subscripts denote scoring interval: 24, 48, or 96 h; f, final score (7 days).

Two requirements must be met before a tentative rating may become final. First, the mean total score for the 7-day scoring interval may not exceed 20 points if the rating is to be maintained. Second, individual total scores for at least 60% of the rabbits should be 10 points or less and in no case may any individual rabbit's total score exceed 30. If either or both of these requirements are not met, then the "tentative rating" must be raised one level, and the higher level becomes the "final rating."

NATIONAL ACADEMY OF SCIENCES (NAS) METHOD BASED ON SEVERITY AND PERSISTENCE²⁸

This descriptive scale, adapted from work conducted by Green et al.,²⁹ attaches significance to the persistence and reversibility of responses. It is based on the most severe response observed in a group of animals rather than the average response.

Inconsequential or Complete Lack of Irritation

Exposure of the eye to a material under the specified conditions causes no significant ocular changes. No staining with fluorescein can be observed. Any changes that occur clear within 24 h and are no greater than those caused by isotonic saline under the same conditions.

Moderate Irritation

Exposure of the eye to the material under the specified conditions causes minor, superficial, and transient changes of the cornea, iris, or conjunctiva as determined by external or slit lamp examination with fluorescein staining. The appearance at the 24 h or subsequent grading interval of any of the following changes is sufficient to characterize a response as moderate irritation: opacity of the cornea (other than a slight dulling of the normal luster), hyperemia of the iris, or swelling of the conjunctiva. All observations resolve within 7 days.

Substantial Irritation

Exposure of the eye to the material under the specified conditions causes significant injury to the eye, such as loss of

the corneal epithelium, corneal opacity, iritis (other than a slight injection), conjunctivitis, pannus, or bullae. The effects resolve within 21 days.

Severe Irritation or Corrosion

Exposure of the eye to the material under the specified conditions results in the same types of injury as in the previous category and in significant necrosis or other injuries that adversely affect the visual process. Injuries persist for 21 days or more.

MODIFIED NAS METHOD DEVELOPED

BY ALLIEDSIGNAL, INC.³⁰

This classification scheme helps distinguish mildly irritating substances from moderately irritating substances, as well as identifying strongly and severely irritating substances. It is based on the most severe ocular response observed in a group of animals, rather than the average response, and on the persistence of the response.

Nonirritation

Exposure of the eye to the material under the specified conditions causes no ocular changes. No tissue staining with fluorescein is observed. Slight conjunctival injection (grade 1; some vessels definitely injected) that does not clear within 24 h is not considered a significant change. This level of change is inconsequential as far as representing physical damage to the eye and can be seen to occur naturally for unexplained reasons in otherwise normal rabbits.

Mild Irritation

Exposure of the eye to the material under the specified conditions causes minor and/or transient changes as determined by external or slit lamp examination or fluorescein staining. No opacity, ulceration, or fluorescein staining of the cornea (except for staining that is characteristic of normal epithelial desquamation) are observed at any grading interval. The appearance of any of the following changes is sufficient to characterize a response as mild irritation:

- Grade 1 hyperemia of the iris that is observed at 1 h, but resolves by 24 h
- Grade 2 conjunctival hyperemia (redness) that is observed at 1, 24, and/or 48 h, but resolves by 72 h
- Grade 2 conjunctival chemosis (swelling) that is observed at 1 h, but diminishes to grade 1 or 0 by 24 h; or Grade 1 conjunctival chemosis that is observed at 1 and/or 24 and/or 48 h, but resolves by 72 h

Moderate Irritation

Exposure of the eye to the material under the specified conditions causes major ocular changes as determined by external or slit lamp examination or fluorescein staining. The appearance of any of the following changes is sufficient to characterize a response as moderate irritation:

- Opacity of the cornea (other than slight dulling of the normal luster) is observed at any observation period, but resolves by day 7.

- Ulceration of the cornea (absence of a confluent patch of corneal epithelium) is observed at any observation period, but resolves by day 7.
- Fluorescein staining of the cornea (greater than that which is characteristic of normal epithelial desquamation) is observed at 1, 2, 3, and/or 4 days, but no staining is found by day 7.
- Grade 1 or 2 hyperemia of the iris (circumcorneal injection, congestion) is observed and persists to 24 h or longer, but resolves by day 7.
- Grade 2 conjunctival hyperemia is observed and persists to at least 72 h, but resolves by day 7; or Grade 3 conjunctival hyperemia is observed at any observation period, but resolves by day 7.
- Grade 1 or greater conjunctival chemosis is observed and persists to 72 h or longer, but resolves by day 7.

Strong Irritation (Clearing within 21 Days)

Exposure of the eye to the material under the specified conditions results in the type of injury described in the former category, but the effects (possibly including pannus or bullae) heal or clear within 21 days.

Severe Irritation (Persisting for 21 Days) or Corrosion

Exposure of the eye to the material under the specified conditions results in the type of injury described in the two former categories, but causes significant tissue destruction (necrosis) or injuries that probably adversely affect the visual process. The effects of the injuries persist for at least 21 days.

CATEGORIZATION OF SUBSTANCES USING THE SLIT LAMP BIOMICROSCOPE AND FLUORESCEIN

Site	"Accept"	"Accept with Caution"	"Probably Injurious to Human Eyes"
Conjunctiva	Hyperemia without chemosis	Chemosis, less than 1 mm at the limbus	Chemosis, greater than 1 mm at the limbus
Cornea	Staining, corneal stippling ^a without confluence at 24 h	Confluence ^b of staining at 24–48 h	Staining with infiltration or edema
Anterior chamber	0	0	Flare ^c (visibility of slit beam; rubeosis of iris)

Sources: Beckley, J.H. et al., *Toxicol. Appl. Pharmacol.*, 15, 1, 1969; Environmental Protection Agency, Guidance for evaluation of eye irritation testing, Hazard Evaluation Division Standard Evaluation Procedures, EPA-540/09-88-105, Washington, DC, 1988.

^a Corneal stippling: multiple discrete punctate irregularities in the corneal epithelial layer, which retain fluorescein.

^b Confluence: uniform zones for fluorescein retention larger than 1 mm in diameter.

^c Flare: Tyndall effect in a beam traversing the aqueous humor.

CATEGORIZATION AND LABELING OF PESTICIDES³³

Label Statements Regarding Eye Irritation Hazards due to Pesticides

Toxicity Category	Signal Word	Skull and Crossbones and "Poison" Required	Precautionary Statement	Practical Treatment
I. Corrosive (irreversible destruction of ocular tissue), corneal involvement, or irritation persisting for more than 21 days.	Danger	No	Corrosive. ^a Causes irreversible eye damage. Harmful if swallowed. Do not get in eyes or on clothing. Wear goggles, face shield, or safety glasses. ^b Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash before reuse.	<i>If in eyes:</i> Flush with plenty of water. Get medical attention. <i>If swallowed:</i> Drink promptly a large quantity of milk, egg whites, gelatin solution, or, if these are not available, drink large quantities of water. Avoid alcohol. <i>Note to physician:</i> Probable mucosal damage may contraindicate the use of gastric lavage.
II. Corneal involvement or irritation clearing in 21 days or less.	Warning	No	Causes substantial but temporary eye injury. Do not get into eyes or on clothing. Wear goggles, face shield, or safety glasses. ^b Harmful if swallowed. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash before reuse.	<i>If in eyes:</i> Flush with plenty of water. Get medical attention. <i>If swallowed:</i> Drink promptly a large quantity of milk, egg whites, gelatin solution, or, if these are not available, drink large quantities of water. Avoid alcohol.

(continued)

Toxicity Category	Signal Word	Skull and Crossbones and "Poison" Required	Precautionary Statement	Practical Treatment
III. Corneal involvement or irritation clearing in 7 days or less.	Caution	No	Causes (moderate) eye injury (irritation). Avoid contact with eyes or clothing. Wash thoroughly with soap and water after handling.	<i>If in eyes:</i> Flush with plenty of water. Get medical attention if irritation persists.
IV. Minimal effects clearing in less than 24 h.	Caution	No	None required.	None required.

^a The term "corrosive" may be omitted if the product is not actually corrosive.

^b Choose appropriate form of eye protection. Recommendation for goggles or face shield is more appropriate for industrial, commercial, or nondomestic uses. Safety glasses may be recommended for domestic or residential use.

CONSUMER PRODUCT SAFETY COMMISSION, FEDERAL HAZARDOUS SUBSTANCES ACT (FHSA) REGULATIONS FOR CLASSIFYING AN EYE IRRITANT (16 CFR 1500.42)

Ocular reactions to a test substance are examined and scored in six albino rabbits. An animal is considered as exhibiting a *positive reaction* if the test substance produces any of the following ocular tissue responses 24, 48, or 72 h after instillation:

Ocular Tissue	Positive Response
Cornea	Ulceration of the cornea (other than a fine stippling) or opacity (other than slight dulling of normal luster)
Iris	Inflammation (other than a slight deepening of the folds or rugae, or a slight circumcorneal injection of the blood vessels)
Conjunctivae	An obvious swelling with partial eversion of the lids or diffuse crimson-red with individual vessels not easily discernible

Classification of a test substance is based on the number of animals exhibiting a positive reaction.

Test Group Result (<i>n</i> = 6)	FHSA Classification
4–6 animals exhibit a positive reaction	Eye irritant
2–3 animals exhibit a positive reaction	Inconclusive ^a
0–1 animals exhibit a positive reaction	Nonirritant

^a If two to three animals exhibit a positive reaction, the test is repeated using six new animals. If three or more animals in the second test exhibit a positive reaction, the test substance is classified as an "eye irritant." If one to two animals in the second test exhibit a positive reaction, a third test is conducted using new animals. If one or more animals in the third test exhibit a positive reaction, the test substance is classified as an "eye irritant."

SPECIALIZED TECHNIQUES USED TO EXAMINE THE EYE FOR TOXIC EFFECTS

Technique	Description	References
Slit lamp biomicroscopy	Used as a visual aid to evaluate the external features of the eye and the anterior portion of the globe (conjunctiva, cornea, iris, lens, anterior portion of the vitreous)	[25,26,34,35]
Direct ophthalmoscopy	Used as a monocular visual aid to evaluate the ocular media and fundus	[36]
Pachymetry	Used to measure the degree of corneal swelling	[37–41]
Electroretinography	Used to determine diffuse retinal damage and to evaluate the functional integrity of the retina when fundoscopic viewing is impaired due to lens opacification (the technique measures the normal change in electrical potential of the eye caused by a diffuse flash of light)	[36]
Specular microscopy	Used to evaluate the integrity of the corneal endothelium	[42]
Scheimpflug photography	Used to analyze and document changes in lens transparency (cataract development)	[43]
Tonometry	Used to measure intraocular pressure of the eye (both contact and noncontact techniques are available)	[44–47]

PROPOSED TIER APPROACHES TO EYE IRRITATION TESTING (FIGURES 4.12 AND 4.13)

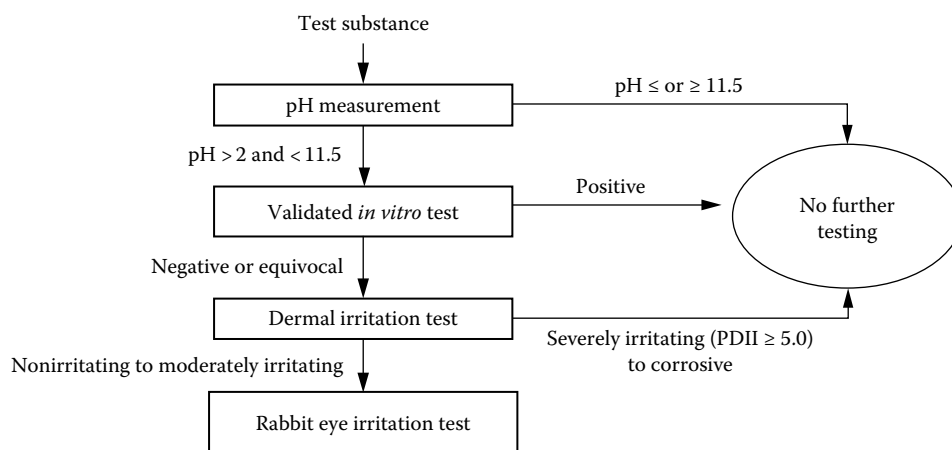


FIGURE 4.12 Schematic screening procedure to assess a test substance's irritation potential before conducting an eye irritation test. (From Reinhardt, C.A., Pelli, D.A., and Zbinden, G., Interpretation of cell toxicity data for the estimation of potential irritation, *Food Chem. Toxicol.*, 23, 247, 1985.)

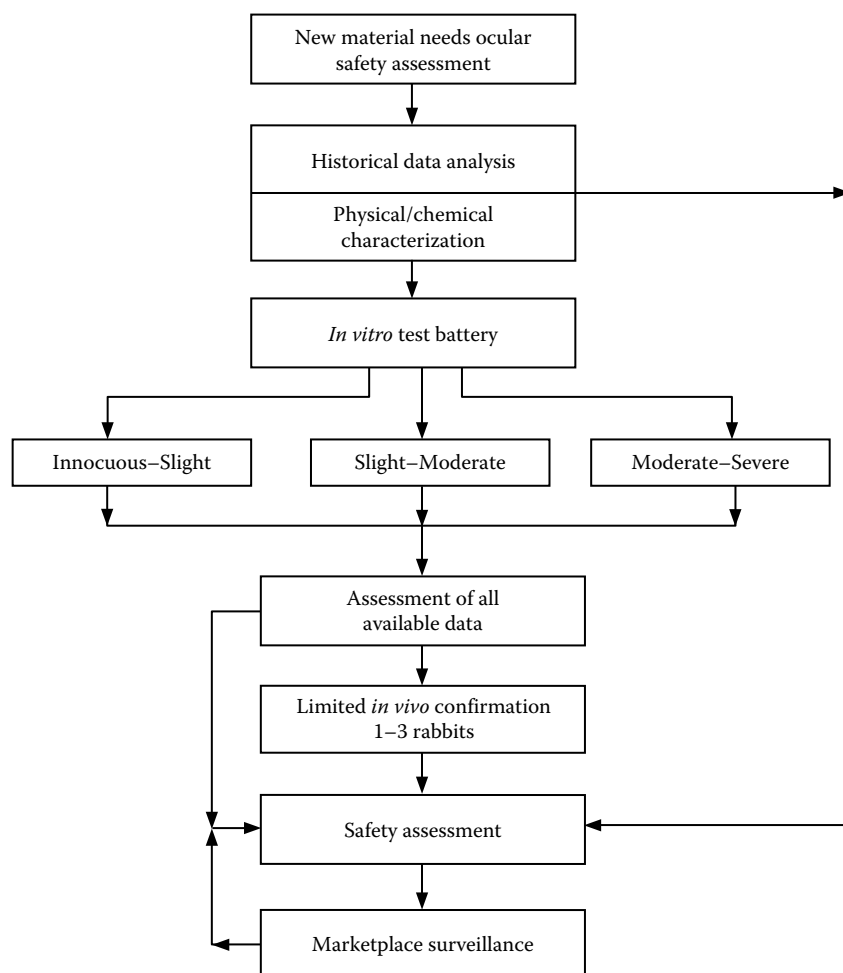


FIGURE 4.13 Diagrammatical representation of how *in vitro* alternatives may be incorporated into the ocular safety assessment process. Initially, all previous testing data and physical chemical characteristics are evaluated. If necessary, the materials are then evaluated in a battery of *in vitro* assays. Following *in vitro* testing, all data are assessed again. Then either a safety assessment would be made or an *in vivo* test would be performed in a limited number of animals before making the final safety assessment. (From Hobson, D.W., *Dermal and Ocular Toxicology, Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.)

OCULAR ADVERSE EFFECTS OF CHEMICAL SUBSTANCES

Tables 4.9 through 4.20 present lists of chemicals that are capable of producing certain ocular adverse effects. The tables were developed from information found in Grant's *Toxicology of the Eye*,⁴⁹ an excellent reference on chemicals, drugs, plants, toxins, and venoms, and their effects on the eyes or vision.

TABLE 4.9
Possible Adverse Corneal Effects

Chemical/Drug	Adverse Effects
Beryllium poisoning	Corneal calcification (band keratopathy)
Calcium hydroxide burn	
Phenylmercuric nitrate	
Polyethylene sulfonic acid	
Vitamin D poisoning	
Allyl alcohol	Corneal epithelial edema (painless) with delayed onset of haloes from local action
Diethylamine	
Diethyl diglycolate	
Diisopropylamine	
Dimethylamine	
Dimethylaminopropylamine	
Dimethyl diglycolate	
Ethylenediamine	
N-Ethylmorpholine	
N-Ethylpiperidine	
N-Methylmorpholine	Corneal epithelial injury (painful) with delayed onset from local action
Morpholine	
tert-Octylamine	
Tetramethylbutanediamine	
Tetramethylethylenediamine	
Triethylenediamine	
Allyl alcohol	
pAnisoyl chloride	
Butyl amine	
Cardiac glycosides	
Colchicine	Corneal opacities from systemic drugs (animals)
Diazomethane	
Dichlorobutanes	
Diethylamine	
Digitalis glycosides	
Diisopropylamine	
Dimethylaminopropylamine	
Dimethylphosphorochloridothionate	
Dimethyl sulfate	
Diphenylcyanoarsine	
Diving mask defogger	Corneal scarring, late (humans)
Dyes (cationic)	
Emetine	
Erythrophleine	
Ethylene oxide	
Ethylenimine	
Euphorbias	
Fish (decomposing)	
Formaldehyde	
Hydrogen sulfide	

TABLE 4.9 (continued)
Possible Adverse Corneal Effects

Chemical/Drug	Adverse Effects
Hypochloriteammonia mixtures	Corneal epithelial deposits from systemic drugs (humans)
Ipecac	
Manchineel	
Methyl bromide	
Methyl chloroacrylate	
Methyl dichloropropionate	
Methyl fluorosulfate	
Methyl silicate	
Mustard gas	
Mustard oil	
Nitrosomethyl urethane	Corneal opacities from systemic drugs (animals)
Osmic acid	
Oxalyl chloride	
Podophyllum	
Poison ivy	
Squill	
Sulfur	
Surfactants	
Triacetoxanthracene	
Trimethyl siloxane	
Vinblastine	Corneal scarring, late (humans)
Amiodarone	
Amodiaquine	
Bismuth subnitrate	
Chloroquine	
Chlorpromazine	
Clofazimine	
Fluphenazine	
Gold	
Hydroxychloroquine	
Isotretinoin	Corneal opacities from systemic drugs (animals)
Mepacrine	
Monobenzene	
Perhexiline	
Tilorone	
Triparanol	
BA 6650	
Carbutamide	
Chlorpropamide	
1,2-Dibromoethane	
1,2-Dichloroethane	Corneal scarring, late (humans)
Dichloronitroaniline	
Dimethylhydrazine	
Epinephrine	
Iminodipropionitrile	
Isotretinoin	
Phthalofyne	
Practolol	
Tolbutamide	
Aniline	
Hydroquinone	
Mustard gas	

TABLE 4.10
Possible Adverse Corneal and Conjunctival Effects

Chemical/Drug	Adverse Effects
Acetone	Burns (humans)
Alcohol	
Ammonia	
Benzalkonium chloride	
Benzene	
Brake fluid	
Brilliant green	
Calcium hydroxide	
Castor beans (ricin)	
<i>a</i> -Chloroacetophenone	
<i>o</i> -Chlorobenzylidene malononitrile	
Chlorobromomethane	
Chlorobutanol	
2-Chloroethanol	
Chloroform	
Chrysarobin (chrysophanic acid)	
Clove oil	
Croton oil	
Crystal violet	
2-Cyanoacrylic acid esters	
Cytarabine	
Dibenzoxazepine	
Dibutyl phthalate	
Dieffenbachia juice	
Digitoxin	
Digoxin	
Dimethyl phthalate	
Dimethyl sulfate	
Emetine hydrochloride	
Euphorbia latex	
Formaldehyde	
Gentian violet	
Hydrochloric acid	
Hydrogen peroxide	
Hydrogen sulfide	
Hydroquinone-benzoquinone	
Iodine vapor	
Isopropyl alcohol	
Lewisite	
Mustard gas	
Mustard oil	
Nitrogen mustards	
Osmium tetroxide	
Podophyllum	
Potassium permanganate	
Propylene imine	
Silver nitrate	
Spitting Cobra venom	

TABLE 4.10 (continued)
Possible Adverse Corneal and Conjunctival Effects

Chemical/Drug	Adverse Effects
Soap	
Sodium hydroxide	
Styrene	
Sulfur dioxide	
Trichloroacetic acid	
Trichloroethylene	
Urea	
Vinblastine	
Zinc chloride	

TABLE 4.11
Possible Adverse Conjunctival Effects

Chemical/Drug	Adverse Effects
Allyl cyanide (rats)	Inflammation from systemic substances
Aminosalicylic acid	
Arsenic (inorganic)	
Arsphenamine	
Barbiturates	
Bromide	
Chloral hydrate	
Chlorambucil	
Chlorpropamide	
Cyclophosphamide	
Cytarabine	
Dixyrazine	
Ethylphenylhydratoin	
Gold	
Hexachlorobenzene	
Hypericum	
Isotretinoin	
Lantana (animals)	
Methotrexate	
Methyldopa	
Noramidopyrine	
Novobiocin	
Oxprenolol	
Penicillamine	
Phenazone	
Phenolphthalein	
Phenazopyridine (dogs)	
Phensuximide	
Phenylbutazone	
Phenytoin	
Phthalofyne	
Practolol	
Sulfadiazine	
Sulfamerazine	
Sulfarsphenamine	
Sulfathiozole	

TABLE 4.12
Possible Adverse Lens Effects

Chemical/Drug	Adverse Effects
Acetaminophen (A)	Opacity (cataract) from systemic administration or exposure
Alloxan (A)	
Allyl cyanide (A)	
Aminotriazole (A)	
Arabinose (A)	
5-Aziridino-2, 4-dinitrobenzamide (A)	
Bis-(phenylisopropyl)-piperidine and UVA (H)	
Bleomycin (A)	
Boron hydride disulfide (A)	
Bromodeoxuridine (reversible) (A)	
Busulfan (A, H)	
Carbutamide (A)	
Chlorphentermine (A)	
Chlorophenylalanine (A)	
Chlorpropamide (A)	
Clomiphene (A)	
Cobalt chloride (A)	
Corticosteroids (H)	
Decahydronaphthalene (A)	
Diazcholesterol (A)	
Diazoxide (reversible) (A)	
Dichlorisone (H)	
Dichloroacetate (reversible) (A)	
Diethylaminoethoxyandrostenone (A)	
4(<i>p</i> Dimethylaminostyryl)quinoline (A)	
Dimethylnitroquinoline (A)	
Dimethyl terephthalate (A)	
Dinitroresol (A, H)	
Dinitrophenol (A, H)	
Diquat (A)	
Disophenol (A)	
Dithizone (A)	
Epinephrine (A)	
Galactose (A, H)	
Hematoporphyrin (A)	
Hygromycin B (A)	
ICI 33828 (A)	
Iodoacetate (A)	
Methoxsalen and UVA (A)	
Methyl dichlorisone (H)	
Mimosine (A)	
Miotics (H)	
Mirex (A)	
Mitotane (H)	
Nafoxidine (A)	
Naphthalene (A)	

TABLE 4.12 (continued)
Possible Adverse Lens Effects

Chemical/Drug	Adverse Effects
2-Naphthol (A)	Lens deposits or discoloration
1,2-Naphthoquinone (A)	
Nitrogen mustard (A)	
Nitroquinolones (A)	
Opiates (A)	
Petroleum fraction (A)	
Phenelzine and serotonin (A)	
3-(2-Phenyl)-hydrazopropionitrile (A)	
2-(4-Phenyl-L-piperazinylmethyl)-cyclohexanone (A)	
(4'-Pyridyl)L-piperazine derivatives (A)	
Pyrithione (A)	
Selenium (A)	
SQ 11290 (A)	
Streptomycin (A)	
Streptozotocin (A)	
Sulfaethoxypyridazine (A)	
Tetrahydronaphthalene (A)	
Thallium (A)	
Tolbutamide (A)	
Tretamine (A)	
Triaziquone (A)	
Trinitrotoluene (H)	
Triparanol (A, H)	
Verapamil (A)	
Xylose (A)	
Amiodarone (H)	
Chlorpromazine (A) (H)	
Copper (H)	
Fluphenazine (H)	
Iprindole (A)	
Iron (H)	
Mercury (H)	
Phenylmercuric salts (H)	
Silver (H)	

Note: A: animals; H: humans.

TABLE 4.13
Possible Adverse Eyelid Effects

Chemical/Drug	Adverse Effects
Amphetamine	Lid retraction
Cocaine	
Levodopa	
Methoxamine	
Phenylephrine	
Barbiturates	Ptosis of eyelids (topical or systemic substances)
Botulism	
Bretylium	
Chloral hydrate	
Chloralose	
Conhydrine	
Coniine	
Corticosteroids	
Curare	
Gelsemium sepervirens	
Guanethidine	
Levodopa	
Mephesisin	
Methylpentynol	
Pelletierine	
Penicillamine	
Phenoxybenzamine	
Primidone	
Reserpine	
Snake venoms	
Spider venoms	
Sulfonal	
Tetraethylammonium	
Thallium	
Trichloroethylene decomposition	
Trimethadione	
Vincristine	

TABLE 4.14
Possible Adverse Retinal Effects

Chemical/Drug	Adverse Effects
Acridine (A)	Retinal edema from systemic administration
Ammi majus seeds (H)	
Chloramphenicol (H)	
Cobalt (A)	
Cyanide (A)	
Dithizone (A)	
Ergotamine (H)	
Ethyl hydrocuprein (H)	
Fluoride (A)	
Glue sniffing (H)	
Glutamate (A)	
Helichrysum (A)	
Iminodipropionitrile (A)	
Iodate (A) (H)	
Iodoquinol (H)	
Methanol (H)	
Naphthalene (A)	
Optochin (H)	
Phosphorus (A)	
Quinine (H)	
Pyrrhione (A)	
Radiopaque media (H)	
Streptomycin (A)	
Triaziquone (A)	
Acetylphenylhydrazine (H)	Retinal hemorrhages from systemic administration
Alloxan (A)	
Arsphenamine (H)	
Aspirin (H)	
Benzene (H)	
Bicycloheptadiene dibromide (H)	
Carbenoxolone (H)	
Carbon disulfide (H)	
Carbon monoxide (H)	
Chloramphenicol (H)	
Dapsone (H)	
Desoxycortone acetate (A)	
Dextran (A)	
Dicumarol (H)	
Diodone (H)	
Diquat (A)	
Dithizone (A)	
Epinephrine (A)	
Ethambutol (H)	
Hexachlorophene (H)	
Iodoform (H)	
Isotretinoin (H)	
Lead (H)	
Licorice (H)	
Methaqualone (H)	
Methyl bromide (H)	

TABLE 4.14 (continued)
Possible Adverse Retinal Effects

Chemical/Drug	Adverse Effects
Miotane (H)	
Naphthalene (A)	
2(2Naphthyl)oxy ethanol (A)	
Phenprocoumon (H)	
Phenylbutazone (H)	
Phosphorus (A)	
Potato leaf smoking (H)	
Pyrrhione (A)	
Radiopaque media (H)	
Snake venoms (H)	
Sulfanilamide (H)	
Sulfathiazole (H)	
Trichloroethylene decomposition (H)	
Triethyl tin (H)	
Vitamin A (H)	
Warfarin (H)	
Amiodarone (A, H)	Retinal lipidosis (phospholipidosis) from systemic administration
AY 9944 (A)	
Chlorcyclizine (A)	
1-Chloroamitriptyline (A)	
Chloroquine (A)	
Clomipramine (A)	
Diethylaminoethoxyhexestrol (A)	
Dithiozone (A)	
Imipramine (A)	
Iprindole (A)	
Perhexilene (A)	
Triparanol (A)	
Ammeline (A)	
Ammi majus seeds (A)	
Aramite (A)	
Benzoic acid (A)	
Bracken fern (A)	
Bromoacetate (A)	
Cardiac glycosides (H)	
Colchicine (A)	
Diaminodiphenylmethane (A)	
Digitalis (H)	
Digitoxin (H)	
Ethylenimine	
Ethylhydrocupreine (A)	
Fluorescein (with light) (A)	
Fluoride (A)	
Furmethonol (H)	
Halothane (A)	
Helichrysum (A)	
Hematoporphyrin (with light) (A)	
Hexachlorophene (A)	
Iodate (A)	

(continued)

TABLE 4.14 (continued)
Possible Adverse Retinal Effects

Chemical/Drug	Adverse Effects
Iodoacetate (A)	
Methylazoxymethanol acetate (A)	
<i>N</i> -Methyl- <i>N</i> -nitrosourea (A)	
2-Naphthol (A)	
P-1727 (A)	
Quinine (A, H)	
Quinoline (A)	
Sodium azide (A)	
<i>Stypantra imbricata</i> (A)	
Sucrose (A)	
Urethane (A)	
Aspidium (A)	Retinal vessel narrowing from systemic administration
Diaminodiphenoxypentane (A)	
Ergotamine (H)	
Ethylenimine (A)	
Ethylhydrocupreine (H)	
Eucupine (H)	
Iron (H)	
Lead (A)	
Oxygen (A) (H)	
P-1727 (A)	
Quinine (H)	
Ammi majus seeds (A)	Retinal ganglion cell damage by systemic administration
Arsanilic acid (A, H)	
Aspidium (A)	
Carbon dioxide (H)	
Carbon disulfide (A)	
Chloramphenicol (H)	
Cinchona derivatives (A)	
Cysteine (A)	
Ergot (A)	
Ethylhydrocupreine (A, H)	
Glutamate (A)	
Locoweed (A)	
Methanol (H)	
Quinine (A, H)	
Quinoline (A)	
Swainsona plants (A)	
Tellurium (A)	
Thallium (H)	
Vincristine (H)	
Alloxan (A)	Retinal pigment epithelial changes from systemic administration
Aminophenoxyalkanes (A)	
Ammeline (A)	
Ammi majus seeds (A)	
Amopyroquin (A)	
Aspartate (A)	
Bilirubin (A)	
Bromoacetate (A)	
Cephaloridine (A)	
Chloroquine (A, H)	
Cobalt (A)	
Colchicine (A)	
Deferoxamine (H)	

TABLE 4.14 (continued)
Possible Adverse Retinal Effects

Chemical/Drug	Adverse Effects
Diaminodiphenoxyheptane (A)	
Diaminodiphenoxypentane (A)	
Diaminodiphenylmethane (A)	
Dibutyl oxalate (A)	
Dihydrodihydroxynaphthalene (A)	
Dithizone (A)	
Epinephrine (A)	
Ethambutol (high dose) (H)	
Ethylenimine (A)	
Ethylhydrocupreine (H)	
Fluoride (A)	
Glutamate (A)	
Hydroxychloroquine (H)	
Iminodipropionitrile (A)	
Iodate (A, H)	
Iodoacetate (A)	
Isopropylhydroxybenzylpyrazolopyrimidine (A)	
Lead (A)	
Mesidine (A)	
<i>N</i> -Methyl- <i>N</i> -nitrosourea (A)	
Naphthalene (A)	
Naphthoquinone (A)	
Naphthyl benzoate (A)	
Nitrofurazone (A)	
Ouabain (A)	
Oxygen (A, H)	
Penicillamine (H)	
Pheniprazine (A)	
Phenylhydrazine (A)	
Phlorizin (A)	
Phosphorus (A)	
Piperidychlorophenothiazine (A, H)	
Quinine (H)	
Quinoline (A)	
Sodium azide (A)	
Sparsomycin (H)	
Tetrahydronaphthalene (A)	
Thioridazine (A, H)	
Toxotoxin (A)	
Trenimon (A)	
Triaziquone (A)	
Trifluoromethylphenylisopropylamine (A)	
Urethane (A)	
Vinblastine (A)	
Vincristine (A)	
Vitamin A (A)	
Acetazolamide (H)	Electroretinogram altered by systemic administration
Aldrin (A)	
4Aminobutyric acid (A)	
Aminophoxyalkanes (A)	
Ammeline (A)	
Ammonium poisoning (A)	
Amodiaquine (H)	
Amyl acetate (A)	
Aramite (A)	

TABLE 4.14 (continued)
Possible Adverse Retinal Effects

Chemical/Drug	Adverse Effects
Aspartate (A)	
Barbiturates (A)	
Befunolol (A)	
Carbaryl (A)	
Carbon disulfide (A)	
Carbon monoxide (A)	
Cardiac glycosides (A)	
Chloramphenicol (H)	
Deferoxamine (H)	
2-Deoxyglucose (A)	
Desipramine (A)	
Diaminodiphenoxypentane (A)	
Digitalis (H)	
Digoxin (A, H)	
Dithizone (A)	
Epinephrine (A)	
Ethambutol (A)	
Fluoride (A)	
Formaldehyde (A)	
Glucose 6phosphate (A)	
Glutamate (A)	
Halothane (H)	
Hydroxychloroquine (A, H)	
Iminodipropionitrile (A)	
Iodate (A)	
Iodoacetate (A)	
Methanol (H)	
Mitomycin C (A)	
Nitrofurazone (A)	
Ouabain (A)	
Oxygen (A) (H)	
Oxypertine (A)	
Piperidylchlorophenothiazine (A)	
Quinine (A, H)	
Rifampin (A)	
Sodium azide (A)	
Styrene (A)	
Sucrose (A)	
Thallium (A)	
Trimethadione (A, H)	
Urethane (A)	
Vincristine (H)	
Vitamin A (A)	
Amoproxan	Central (or cecentral) scotomas from systemic administration (humans)
Caramiphene	
Carbon disulfide	
Chloramphenicol	
Chloroquine	
Chlorpropamide	
Clomiphene	
Digitalis	
Diogitoxin	
Digoxin	
Dinitrobenzene	
Dinitrochlorobenzene	
Dinitrotoluene	

TABLE 4.14 (continued)
Possible Adverse Retinal Effects

Chemical/Drug	Adverse Effects
Disulfiram	
Ergotamine	
Emetine	
Ethambutol	
Ethchlorvynol	
Ethyl alcohol	
Ethylene glycol	
Flumequine	
Ibuprofen	
Iodate	
Iodoform	
Isoniazid	
Lead	
Methanol	
Methyl bromide	
Minoxidil	
Octamoxin	
Pheniprazine	
Plasmocid	
Streptomycin	
Sulfonamides	
Tetraethyl lead	
Thallium	
Thiacetazone	
Tobacco smoking	
Trichloroethylene decomposition	
Wasp sting	
Amodiaquine	Peripheral visual field constriction from systemic administration (humans)
Arsacetin	
Arsanilic acid	
Bee sting	
Botulism toxin	
Carbon dioxide	
Carbon monoxide	
Chloramphenicol	
Cortex granati	
Dionitrochlorobenzene	
Emetine	
Ethambutol	
Ethylhydrocupreine	
Ethylmercuritoluenesulfonanilide	
Eucupine	
Iodate	
Methylmercury compounds	
Methanol	
Naphthalene	
Orsudan	
Oxygen	
Pheniprazine	
Piperidylchlorophenothiazine	
Quinine	
Trichloroethylene decomposition	
Tryparsamide	

Note: A: animals; H: humans.

TABLE 4.15
Possible Adverse Optic Nerve Effects

Chemical/Drug	Adverse Effects
Acetarsone (H)	Optic nerve atrophy from systemic administration
Acetylarson (H)	
Antimony potassium tartrate (H)	
Arsacetin (H)	
Arsanilic acid (A, H)	Optic neuropathy from systemic administration
Aspidium (A, H)	
Bee sting	
Brayera (H)	
Broxyquinoline (H)	
Carbon dioxide (H)	
Carbon disulfide (H)	
Caster beans (H)	
Chloramphenicol (H)	
Clioquinol (H)	
Cortex granati (H)	
Dapsone (H)	
Dinitrobenzene (H)	
Dinitrochlorobenzene (H)	
Ethambutol (H)	
Ethyl hydrocuprein (H)	
Ethylmercuritoluenesulfonanilide (H)	
Eucupine (H)	
Finger cherries (H)	
Formic acid (A)	
Halquinols (H)	
Hexachlorophene (A) (H)	
Hexamethonium (H)	
Iodoform (H)	
Iodoquinol (H)	
Isoniazid (H)	
Lead (H)	
Methanol (A, H)	
Octamoxin (H)	
Pheniprazine (H)	
Plasmocid (H)	
Quinine (H)	
Solvent sniffing (H)	
Thallium (H)	
Trichloroethylene decomposition (H)	
Triethyl tin (H)	
Tryparsamide (H)	
Vincristine (H)	
Acetarsone (H)	Optic neuropathy from systemic administration
Acetylarson (H)	
Acrylamide (A)	
Antirabies vaccine (H)	
Arsacetin (H)	
Arsanilic acid (A, H)	
Aspidium (H)	
Bee sting (H)	
Botulism (H)	
Carbon disulfide (H)	

TABLE 4.15 (continued)
Possible Adverse Optic Nerve Effects

Chemical/Drug	Adverse Effects
Cassava (H)	Papilledema (swelling of the optic disk) from systemic administration
Chloramphenicol (H)	
Clioquinol (A, H)	
Cyanoacetic acid (A)	
Deferoxamine (H)	
Dinitrobenzene (H)	
Dinitrochlorobenzene (H)	
Dinitrotoluene (H)	
Disulfiram (H)	
Ethambutol (A, H)	
Ethchlorvynol (H)	
Ethylene glycol (H)	
Filicin (A)	
Glutamate (A)	
Helichrysum (A)	
Hexachlorophene (H)	
Iminodipropionitrile (A)	
Indarsol (A)	
Iodoform (H)	
Isoniazid (H)	
Lead (A, H)	
Methanol (H)	
Octamoxin (H)	
Penicillamine (H)	
Perhexiline maleate (H)	
Phosphorus (H)	
Plasmocid (H)	
Sodium azide (A)	
Streptomycin (H)	
Stypantra imbricata (A)	
Sulfonamides (H)	
Tellurium (A)	
Thallium (H)	
Tolbutamide (H)	
Trichloroethylene decomposition (H)	
Triethyl tin (A)	
Trinitrotoluene (H)	
Tryparsamide (H)	
Vincristine (H)	
Antimony potassium tartrate (H)	Papilledema (swelling of the optic disk) from systemic administration
Arsphenamine (H)	
Aspirin (H)	
Bee sting (H)	
Carbenoxolone (H)	
Cephaloridine (H)	
Chlorambucil (H)	
Chloramphenicol (H)	
Chlordecone (H)	
Cisplatin (H)	
Contraceptive hormones (H)	
Corticosteroids (H)	
pDichlorobenzene (A)	

TABLE 4.15 (continued)
Possible Adverse Optic Nerve Effects

Chemical/Drug	Adverse Effects
Dynamite (H)	
Ergotamine (H)	
Ethylene glycol (H)	
Helichrysum (A)	
Hexachlorophene (A, H)	
Isoniazid (H)	
Isotretinoin (H)	
Ketoprofen (H)	
Lead (H)	
Levothyroxine (H)	
Minocycline (H)	
Minoxidil (H)	
Mitotane (H)	
Nalidixic acid (H)	
Nitrofurantoin (H)	
DLPenicillamine (H)	
Penicillin (H)	
Perhexiline maleate (H)	
<i>m</i> -Phenylenediamine (H)	
<i>p</i> -Phenylenediamine (H)	
Phosphorus (H)	
Sulfonamides (H)	
Tetracycline (H)	
Triethyl tin (H)	
Vitamin A (H)	
Aspidium	Retrolbulbar neuritis from systemic administration
Carbon disulfide	
Cassava	
Chloramphenicol	
Deferoxamine	
Dinitrobenzene	
Dinitrochlorobenzene	
Dinitrotoluene	
Disulfiram	
Ethambutol	
Iodoform	
Isoniazid	
Lead	
Octamoxin	
<i>m</i> - or <i>p</i> -Phenylenediamine	
Thallium	
Tolbutamide	
Trichloroethylene decomposition	
Trinitrotoluene	
Chloramphenicol (A)	Optic chiasm injury by systemic administration
Cyanide (A)	
Chloramphenicol (A)	
Cyanide (A)	
Ethambutol (A, H)	
Helichrysum (A)	
Hexachlorophene (H)	
Stypantra imbricata (A)	

TABLE 4.15 (continued)
Possible Adverse Optic Nerve Effects

Chemical/Drug	Adverse Effects
Tellurium (A)	
Triethyl tin (A)	
Vincristine (H)	

Note: A: animals; H: humans.

TABLE 4.16
Possible Adverse Extraocular Muscle Effects

Chemical/Drug	Adverse Effects
Alcuronium	Weakness or paralysis from systemic administration
Amanita phalloides	
Amitriptyline	
Anesthesia, spinal	
Antirabies vaccine	
Arsphenamine	
Barbiturates	
Botulinus toxin	
Carbamazepine	
Curare	
Diazinon	
Ethyl alcohol	
Ethylene glycol	
Furmethonol	
Gelsemium sempervirens	
Hexachlorophene	
Isopentaquine	
Lead	
Minocycline	
Nalidixic acid	
Pamaquine	
Penicillamine	
Pentaquine	
Piperazine	
Plasmocid	
Primidone	
Scorpion venom	
Snake venoms	
Streptomycin	
Sulfonal	
Thallium	
Trichloroethylene decomposition	
Triethyl tin	
Vincristine	
Vitamin A	

TABLE 4.17
Possible Adverse Effects on Intraocular Pressure

Chemical/Drug	Adverse Effects
Acetazolamide (A) (H)	Reduction of intraocular pressure by systemic administration
Alcohol (H)	
Alcuronium (H)	
Aminophylline (H)	
Ascorbic acid (H)	
BA 6650 (A)	
Bromocriptine (H)	
Calcium chloride (A)	
Cardiac glycosides (H)	
Catha edulis (H)	
Chlorpromazine (H)	
Chlorthalidone (H)	
Cholera toxin (H)	
Contraceptive hormones (H)	
Dextran A (H)	
Dibenamine (H)	
Dichlorphenamide (H)	
Digitoxin (H)	
Digoxin (H)	
Dihydroergotoxine (H)	
Ethoxzolamide (H)	
Glucose (H)	
Glycerine (H)	
Iodate (A)	
Iodoacetate (A)	
Isosorbide (H)	
Lanatoside C (A)	
Mannitol (H)	
Meprobamate (H)	
Mercaptomerin (A)	
Mercuderamide (A)	
Methazolamide (H)	
Methyldopa (H)	
Nialamide (A)	
Nitroglycerin (H)	
Ouabain (A)	
Pargyline (H)	
Phentolamine (H)	
Propranolol (H)	
Propylene glycol (H)	
Quinine (H)	
Sodium ascorbate (H)	
Sodium chloride (H)	
Sodium lactate (H)	
Sorbitol (H)	
Thiopental (H)	
Timolol (H)	
Trometamol (H)	
Urea (H)	

Note: A: animals; H: humans.

TABLE 4.18
Possible Adverse Effects on Vision

Chemical/Drug	Adverse Effects
Barbiturates	Cortical blindness (humans)
Carbon monoxide	
Diatrizoate	
Lead	
Lomotil	
Methadone	
Methylergonovine	
Methylmercury compounds	
Vincristine	
Acetyl digitoxin	Color vision alterations from systemic administration
Aconite	
Amodiaquine	
Barbiturates	
Cannabis	
Carbon dioxide	
Carbon disulfide	
Chloramphenicol	
Chlorothiazide	
Contraceptive hormones	
Digitalis	
Digoxin	
Dihydrostreptomycin	
Diphenhydramine theoclate	
Ethambutol	
Furmethanol	
Herbatox	
Ibuprofen	
Lead	
Lysergide	
Nalidixic acid	Altered dark adaption from systemic administration (humans)
Pentylene-tetrazole	
Salicylate	
Carbon dioxide	
Carbon disulfide	
Carbon monoxide	
Deferoxamine	
Digitalis	
Digitoxin	
Halothane	
Indomethacin	Acute transient myopia from systemic administration without cyclotonia or miosis (humans)
Piperidylchlorophenothiazine	
Acetazolamide	
Aminophenazone	
Arsphenamine	
Bendroflumazide	
Chlorothiazide	
Chlorthalidone	
Clofenamide	
Dichlorphenamide	
Ethoxzolamide	
Hydrochlorothiazide	
Isotretinoin	
Neosphenamine	
Phenformin	
Polythiazide	
Prochlorperazine	
Promethazine	
Quinine	
Spironolactone	
Sulfonamides	
Tetracycline	
Trichlormethiazide	

TABLE 4.19
Possible Adverse Irritating Effects

Chemical/Drug	Adverse Effects
Acrolein	Lacrimation from direct exposures
Allyl propyl disulfide	
Bromoacetone	
Bromoacetophenone	
Bromobenzyl cyanide	
Bromomethyl ethyl ketone	
Bromotoluene	
Bromoxylene	
Chloroacetone	
Chloroacetophenone	
Chlorobenzylidene malononitrile	
Chlorosulfonic acid esters	
Cyanic acid	
Cyanogen chloride	
Dibenzoxazepine	
Dibromomethyl ether	
Dichloroformoxime	
Dichloronitroethane	
Diphenylchlorarsine	
Ethylarsine dichloride	
Ethyl benzene	
Ethyl bromoacetate	
Ethyl iodoacetate	
Hexafluoroisopropanol	
Iodotoluene	
Lewisite	
Methyl arsine dichloride	
Methyl iodoacetate	
Methyl vinyl ketone	
Nitrobenzyl chloride	
Nitroethylene	
Onion vapor	
Pelargonic acid morpholide	
Phenylcarbylamine chloride	
Trichloroacetronitrile	
Trichloromethane sulfonyl chloride	
Trichloromethanethiol	
Trichloropyrimidine	
Xylyl bromides	
Xylyl chlorides	
Arsenic, inorganic (H)	Lacrimation with burning or itching sensation from systemic administration
Bethanechol (H)	
Bismuth subnitrate (A)	
Chloral hydrate (H)	
Cyclohexanol (A, H)	
Diazoxide (H)	
Dichlorophenoxy acetic acid (A)	
Dimercaprol (H)	
Dimidium bromide (A, H)	
Diphenylarsenic acid (H)	
Emetine (H)	
Fish (Ciguatera) poisoning (H)	
Herion (H)	
Hexachloronaphthalene (A)	
Hydralazine (H)	
Iodide (H)	
Mercury (acrodynia) (H)	

TABLE 4.19 (continued)
Possible Adverse Irritating Effects

Chemical/Drug	Adverse Effects
Methotrexate (H)	
Morphine withdrawal (H)	
Nicotiazone (H)	
Nitrofurantoin (H)	
Pentazocine withdrawal (H)	
Phthalofyne (A, H)	
Practolol (H)	
Pyrrithione (A)	
Reserpine (H)	
Scorpion venom (H)	
Sulfathiazole (H)	
Tegafur (H)	
Thiacetazone (H)	
Triethyl tin (H)	
Zoxazolamine (H)	

Note: A: animals; H: humans.

TABLE 4.20
Possible Ocular Teratogenesis

Chemical/Drug	Adverse Effects
Alloxan (A)	Abnormalities of the eyes
Aspidium (A)	
Azathioprine (A)	
Busulfan (H)	
Butylated hydroxytoluene (A)	
Caffeine (A)	
Carbutamide (A)	
Chlorambucil (A, H)	
Clomiphene (A)	
Colchicine (A)	
Cyclizine (A)	
Cyclophosphamide (A)	
2,4-Dichlorophenyl- <i>p</i> -nitrophenyl ether (A)	
Felicin (A)	
Heptachlor (A)	
Idoxuridine (A)	
Isotretinoin (A)	
Lysergide (A, H)	
1-Methyl,3-nitro,1-nitroguanidine (A)	
2Naphthol (A)	
Nickel carbonyl (A)	
Quinine (A, H)	
Salicylate (A)	
Thalidomide (A, H)	
Trimethadione (H)	
Trypan blue (A)	
Urethane (A)	
Veratrum californicum (A)	
Vidarabine (A)	
Vinblastine sulfate (A)	
Vitamin A (A)	
Warfarin (H)	

Note: A: animals; H: humans.

REFERENCES

1. Beckley, J.H., Comparative eye testing: man vs. animal, *Toxicol. Appl. Pharmacol.*, 7, 93, 1965.
2. Maurice, D.M. and Giardini, A.A., A simple optical apparatus for measuring the corneal thickness, and the average thickness of the human cornea, *Br. J. Ophthalmol.*, 48, 61, 1951.
3. Marzulli, F.N. and Simon, M.E., Eye irritation from topically applied drugs and cosmetics: preclinical studies, *Am. J. Optom.*, 48, 61, 1971.
4. Gaasterland, D.E., Barranger, J.A., Rapoport, S.I., Girtton, M.E., and Doppman, J.L., Long-term ocular effects of osmotic modification of the blood-brain barrier in monkeys. I. Clinical examinations; aqueous ascorbate and protein, *Invest. Ophthalmol. Visual Sci.*, 24(2), 153, 1983.
5. Kinsey, V.E., Comparative chemistry of aqueous humor in posterior and anterior chambers of rabbit eye, *Arch. Ophthalmol.*, 50, 401, 1953.
6. DeBarnadinis, E. et al., The chemical composition of the human aqueous humor in normal and pathological conditions, *Exp. Eye Res.*, 4, 179, 1965.
7. Graymore, C.N., *Biochemistry of the Eye*, Academic Press, New York, 1970.
8. Davson, H. and Graham, L.T., Comparative aspects of the intraocular fluids, in *The Eye*, Vol. 5, Davson, H., Ed., Academic Press, New York, 1974.
9. Bito, L., Intraocular fluid dynamics. I. Steady-state concentration gradients of magnesium, potassium and calcium in relation to the sites and mechanisms of ocular cation transport processes, *Exp. Eye Res.*, 10, 102, 1970.
10. Kinsey, V.E. and Reddy, D.V.N., Aqueous humor, in *The Rabbit in Eye Research*, Prince, J.H., Ed., Charles C Thomas, Springfield, IL, 1965.
11. Laurent, V.B.G., Hyaluronate in aqueous humour, *Exp. Eye Res.*, 33, 147, 1981.
12. Wegener, J.K. and Moller, P.M., Oxygen tension in the anterior chamber of the rabbit eye, *Acta Ophthalmol.*, 49, 577, 1971.
13. Kleinfeld, O. and Neumann, H.G., Der sauerstoffgehalt des menschlichen kammerwassers, *Klin. Monatsbl. Augenheilkd.*, 35, 224, 1959.
14. Walker, A.M., Comparison of the chemical composition of aqueous humor, cerebrospinal fluid, lymph and blood from frogs, higher animals and man. Reducing substances, inorganic phosphate, uric acid, urea, *J. Biol. Chem.*, 101, 269, 1933.
15. Duke-Elder, Sir S., Physiology of the eye, in *System of Ophthalmology*, Vol. 4, C.V. Mosby, St. Louis, MI, 1968.
16. Dernouchamps, J.P., The proteins of the aqueous humour, *Doc. Ophthalmol.*, 53, 193, 1982.
17. Blogg, J.R. and Coles, E.H., Aqueous humour proteins: A review, *Vet. Bull.*, 40, 347, 1970.
18. Furuichi, C., The influence of various experimental injuries on creatine, creatinine metabolism of aqueous fluid of the rabbit's eye, *Acta Soc. Ophthalmol.*, 65, 561, 1961.
19. McLaughlin, P.S. and McLaughlin, B.G., Chemical analysis of bovine and porcine vitreous humors: Correlation of normal values with serum chemical values and changes with time and temperature, *Am. J. Vet. Res.*, 48, 467, 1987.
20. Nordmann, J., in *Biologie et Chirurgie des Corps Vitre*, Brini, A., Ed., Masson & Cis, Paris, France, 1968.
21. Goldenthal, E.I., Current views on safety evaluation of drugs, *FDA Papers*, 2, 13, 1968.
22. Federal Register, Interagency Regulatory Liaison Group recommended guideline for acute eye irritation testing, National Technological Information Service PB82-117557, 1981.
23. Organization for Economic Cooperation and Development, Acute eye irritation and corrosion, Publication 405, OECD Publishing, 2002.
24. Draize, J.H., Woodard, G., and Calvery, H.O., Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes, *J. Pharmacol. Exp. Ther.*, 82, 377, 1944.
25. Baldwin, H.A., McDonald, T.O., and Beasley, C.H., Slitlamp examination of experimental animal eyes. II. Grading scales and photographic evaluation of induced pathological conditions, *J. Soc. Cosmet. Chem.*, 24, 181, 1973.
26. Hackett, R.B. and McDonald, T.O., Eye irritation, in *Dermatotoxicology*, 4th edn., Marzulli, F.N. and Maibach, H.I., Eds., Hemisphere, New York, 1991.
27. Kay, J.H. and Calandra, J.C., Interpretation of eye irritation tests, *J. Soc. Cosmet. Chem.*, 13, 281, 1962.
28. Committee for the revision of NAS Publication 1138, *Principles and Procedures for Evaluating the Toxicity of Household Substances*, National Academy of Sciences, Washington, DC, 1977.
29. Green, W.R. et al., *A Systematic Comparison of Chemically Induced Eye Injury in the Albino Rabbit and Rhesus Monkey*, The Soap and Detergent Association, New York, 1978, 407.
30. Dunn, B., Toxicology of the eye, in *CRC Handbook of Toxicology*, Derelanko, M.J. and Hollinger, M.A., Eds., CRC Press, Boca Raton, FL, 1995, p. 186.
31. Beckley, J.H., Russell, T.J., and Rubin, L.F., Use of the Rhesus monkey for predicting human response to eye irritants, *Toxicol. Appl. Pharmacol.*, 15, 1, 1969.
32. Environmental Protection Agency, Guidance for evaluation of eye irritation testing, Hazard Evaluation Division Standard Evaluation Procedures, EPA-540/09-88-105, Washington, DC, 1988.
33. Camp, D.D., Labeling requirements for pesticides and devices, *Federal Register*, 49, 188, 1984.
34. McDonald, T.O., Baldwin, H.A., and Beasley, C.H., Slitlamp examination of experimental animal eyes. I. Techniques of illumination and the normal animal eye, *J. Soc. Cosmet. Chem.*, 24, 163, 1973.
35. McDonald, T.O., Kasten, K., Herve, R., Gregg, S., Borgmann, A.R., and Murchison, T., Acute ocular toxicity of ethylene oxide, ethylene glycol, and ethylene chlorohydrin, *Bull. Parenter. Drug Assoc.*, 27, 153, 1973.
36. Chang, D.F., Ophthalmic examination, in *General Ophthalmology*, 12th edn., Vaughan, D., Asbury, T., and Tabbara, K., Eds., Appleton & Lange, Norwalk, CT, 1989.
37. Mishima, S., Corneal thickness, *Survey Ophthalmol.*, 13, 57, 1968.
38. Mishima, S. and Hedbys, B.O., Measurements of corneal thickness with the Haag-Streit pachometer, *Arch. Ophthalmol.*, 80, 710, 1968.
39. Jacobs, G.A. and Martens, M.A., An objective method for the evaluation of eye irritation *in vivo*, *Fd. Chem. Toxicol.*, 27, 255, 1989.
40. Morgan, R.L., Sorenson, S.S., and Castles, T.R., Prediction of ocular irritation by corneal pachymetry, *Food Chem. Toxicol.*, 25, 609, 1987.

41. Kennah, H.E., Hignet, S., Laux, P.E., Dorko, J.D., and Barrow, C.S., An objective procedure for quantitating eye irritation based on changes of corneal thickness, *Fundam. Appl. Toxicol.*, 12, 258, 1989.
42. Leibowitz, H.M. and Laing, R.A., Specular microscopy, in *Corneal Disorders: Clinical Diagnosis and Management*, Leibowitz, H. M., Ed., W.B. Saunders, Philadelphia, PA, 1984.
43. Scheimpflug, T., Der photoperspektograph und seine Anweendung, *Photogr. Korr.*, 43, 516, 1906.
44. Forbes, M., Pico, G. Jr., and Grolman, B., A noncontact applanation tonometer, *Arch. Ophthalmol.*, 91, 134, 1974.
45. Callaway, S., Gazzard, M.F., Price Thomas, D., and Swanston, D.W., The calibration and evaluation of a handheld tonometer as a means of measuring the intraocular pressure in the conscious rabbit, *Exp. Eye Res.*, 15, 383, 1973.
46. Pollack, I.P., Viernstein, L.J., and Radius, R.L., An instrument for constant-pressure tonography, *Exp. Eye Res.*, 29, 579, 1979.
47. Hilton, G.F. and Shaffer, R.N., Electronic applanation tonometry, *Am. J. Ophthalmol.*, 62, 838, 1966.
48. Reinhardt, C.A., Pelli, D.A., and Zbinden, G., Interpretation of cell toxicity data for the estimation of potential irritation, *Food Chem. Toxicol.*, 23, 247, 1985.
49. Grant, W.M., *Toxicology of the Eye*, 3rd edn., Charles C Thomas, Springfield, IL, 1986.
6. Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Edsall, D.J., Holzhutter, H.G., and Liebsch, M., The ECVAM international validation study on in vitro tests for skin corrosivity. 2. Results and evaluation by the Management Team, *Toxicol. In Vitro*, 12, 483–524, 1998.
7. EU, Official Journal of The European Communities L136/91 of 8 June 2000, Method B.40 Skin Corrosion, 2000.
8. OECD, Test Guideline 404. Acute Dermal Irritation/Corrosion, 2000.
9. OECD, OECD Test Guidelines Programme: Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. Held in Solna, Sweden, 22–24 January, 1996 (<http://www.oecd.org/ehs/test/background.htm>).
10. OECD, Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as Endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, November, 1998 (<http://www.oecd.org/ehs/Class/HCL6.htm>).
11. OECD, Guidance document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation. OECD Environmental Health and Safety Publications. Series on Testing and Assessment No. 19, 2000 (<http://www.oecd.org/ehs/test/monos.htm>).
12. Buehler, E.V. and Newmann, E.A., A comparison of eye irritation in monkeys and rabbits, *Toxicol. Appl. Pharmacol.*, 6, 701–710, 1964.
13. Draize, J.H., *Dermal Toxicity. Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*, The Association of Food and Drug Officials of the U.S., 1959, 3rd printing, 1975, pp. 49–52.
14. Draize, J.H. et al., Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes, *J. Pharmacol. Exp. Therap.*, 83, 377–390, 1944.
15. Kay, J.H. and Calandra, J.C., Interpretation of eye irritation tests, *J. Soc. Cosmetic Chem.*, 13, 281–289, 1962.
16. Loomis, T.A., *Essentials of Toxicology*, 3rd edn., Lea & Febiger, Philadelphia, PA, 1978, pp. 226–232.
17. National Academy of Sciences, *Principles and Procedures for Evaluating the Toxicity of Household Substances*, A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC, 1977.
18. World Health Organization, *Part I. Environmental Health Criteria 6. Principles and Methods for Evaluating the Toxicity of Chemicals*, World Health Organization, Geneva, 1978.

FURTHER READING

1. Barratt, M.D., Castell, J.V., Chamberlain, M., Combes, R.D., Dearden, J.C., Fentem, J.H., Gerner, I., Giuliani, A., Gray, T.J.B., Livingston, D.J., Provan, W.M., Rutten, F.A.J.J.L., Verhaar, H.J.M., and Zbinden, P., The integrated use of alternative approaches for predicting toxic hazard, ECVAM Workshop Report 8, *ATLA*, 23, 410–429, 1995.
2. de Silva, O., Cottin, M., Dami, N., Roguet, R., Catroux, P., Toufic, A., Sicard, C., Dossou, K.G., Gerner, I., Schleder, E., Spielmann, H., Gupta, K.C., and Hill, R.N., Evaluation of eye irritation potential: Statistical analysis and tier testing strategies, *Food Chem. Toxicol.*, 35, 159–164, 1997.
3. Worth A.P. and Fentem J.H., A general approach for evaluating stepwise testing strategies, *ATLA*, 27, 161–177, 1999.
4. Young, J.R., How, M.J., Walker, A.P., and Worth, W.M.H., Classification as corrosive or irritant to skin of preparations containing acidic or alkaline substance without testing on animals, *Toxicol. In Vitro*, 2, 19–26, 1988.
5. Neun, D.J., Effects of alkalinity on the eye irritation potential of solutions prepared at a single pH, *J. Toxicol. Cut. Ocular Toxicol.*, 12, 227–231, 1993.

5 *In Vitro* Methods for the Prediction of Ocular and Dermal Toxicity

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INTRODUCTION TO *IN VITRO* TOXICOLOGY

In vitro toxicity methods were first developed to examine the mechanisms underlying actions of chemicals on organs or tissues observed *in vivo*. They were used to isolate the presumed target cells or tissues from the external influences of surrounding tissues or organs. The test chemical could be delivered directly to the cells and the changes in viability or other functional parameters measured. Since the gross effect on the tissue was known from *in vivo* studies, the *in vitro* model or models were designed to elucidate the possible mechanisms by which that change might be induced. As each test system might address only a subset of tissue targets, a battery of assays might be employed to screen for the mode of action for that chemical on the tissue *in vivo*. These initial *in vitro* assay applications were designed to explain the observed action *in vivo* rather than to predict future activities specifically. Although *in vitro* studies of this type remain a major part of cell and molecular biological research, significant advances have been made in the use of *in vitro* assays for predictive toxicology, particularly by the industry for product stewardship and regulatory safety assessments.

Increasingly, *in vitro* methods are being applied prospectively in many fields, including cancer biology, drug discovery, and toxicology. These studies are intended to predict the potential for action of the test material on the target tissue. Studies to assess potential action of the test chemical (for efficacy or hazard assessments) measure changes in the target cells under conditions of known *in vitro* exposure. Such exposure might not be achieved *in vivo* depending on the absorption, distribution, metabolism, and excretion of the chemical. Thus, the primary focus is on the action of individual chemicals/active ingredients under standardized conditions and/or assessment of their relative “potency” in a single cell type. Whereas historically the most common culture format for individual cell types has been the submerged cell monolayer attached to a plastic substrate (e.g., culture flask or multiwell plate), considerable progress has been made in developing and validating

more complex, biologically relevant human cell-based models. For example, hepatocyte culture has progressed from simple cell monolayers that characteristically dedifferentiate and lose their enzymatic functions, to two- and three-dimensional (3D) systems where cell–cell and cell–substrate interactions are optimized to promote development and maintenance of more native functions. Perhaps, the most significant improvements in cell culture systems have been in the development of 3D human tissue reconstructions, especially those that model the complex tissue epithelia and underlying fibroblast scaffolding.¹ In particular, these 3D models (*ex vivo* or engineered tissues) may be the only acceptable systems for evaluating the potential action of topically exposed chemicals and complex formulations on these target tissues. Whereas in monoculture systems one can investigate impacts of actives at the individual cellular level, 3D tissue reconstructions allow one to investigate cellular as well as gross architectural changes in complex differentiated tissue models, and allow one to better model the chemical kinetics following exposure.

Assessment of ocular and dermal toxicity is a standard practice for many types of consumer and industrial formulations and individual chemicals. The development of *in vitro* assays to predict ocular and dermal toxicity has proved to be challenging. Even though the *in vivo* exposures are topical, the exposure kinetics may be quite complex.² Therefore, topical application to tissues (natural or reconstructed) *in vitro* has proved to be the most useful method of testing broad classes of chemicals. Select monolayer cell culture systems have proved valuable for predicting the ocular irritation potential of aqueous-soluble chemicals and product classes, provided that their irritancy potential was not rapidly quenched by aqueous dilution. For example, products containing surfactants may be readily tested in these culture medium-based assays, since surfactants retain their inherent activity in an aqueous environment.³ In contrast, products containing ethanol or acetone may be notably underestimated in these assays since the cytotoxic effects of these small solvents are rapidly diluted to extinction in the

aqueous culture medium.⁴ Furthermore, many other product classes are not amenable to these simpler systems because some of the actives in the formulation may be hydrophobic and thus not adequately bioavailable to the test system. Accordingly, these products should ideally be tested in a tissue-based assay system where the undiluted formulations can be presented to the test system in the same manner as occurs *in vivo* and the effects of the actives appropriately evaluated.^{5,6} These tissues may be short-term *ex vivo* organ cultures or engineered tissue constructs. Complex exposure kinetics, such as dermal penetration and subsequent action of topically applied formulations, require the use of complex systems that can model both the barrier properties of skin and provide the necessary target cells.⁷

This chapter is divided into three parts. This first section, Tables 5.1 through 5.6, provide a general introduction to *in vitro* methods, measures of cellular function, assay end points, design considerations, and controls. The second section, Tables 5.7 through 5.19, address current approaches to assessing ocular irritation potential of chemicals and formulations. The third section, Tables 5.20 through 5.30, focus on the systems to assess action on the dermal tissues. Sample protocol summaries are provided to illustrate different types of assays for ocular and dermal activity. These protocols generally reflect the basic method, which may then be tailored to meet the user's specific needs. Currently, there are several *in vitro* methods that were derived from these basic methods and have subsequently been validated for regulatory safety, classification, and labeling purposes. Those with regulatory applications will be highlighted. Sample data from several common *in vitro* methods are also provided. The historical positive control ranges come from the author's (HR) laboratory and are used to establish the range of acceptable assay performance. Since it is imperative that positive controls are tested each time an assay is performed, these historical control ranges are used to judge the acceptability of each individual trial.

In vitro test systems include target cell cultures or tissues that are isolated and maintained in a supportive culture environment. These tissues may be either *ex vivo* (isolated bovine corneas, isolated chicken eyes, and isolated rabbit eyes) or engineered tissue constructs (human-engineered tissues to model eye [corneal epithelium] or skin). Influences of collateral tissues and/or the systemic environment are eliminated so that primary and secondary effects of the test substance can be more easily determined. Proper selection of the target cell(s)/tissue depends on the specific mode of action and exposure kinetics to be tested. Since cell/tissue homeostasis depends on the culture conditions, careful attention to all facets of test system maintenance and manipulation is essential.⁸ Maintenance of the required cell/tissue differentiation must be demonstrated for each test system. It is now widely accepted that for regulatory classification and labeling of chemicals for skin and eye irritation, no single nonanimal test method will likely meet the full needs for comprehensive irritancy assessment.⁹ Current approaches recognize the strengths of robust full-thickness models for the top-down

assessment of presumed irritants, while highly sensitive epithelial models provide the requisite ability to identify the truly mild and nonirritant (NI) materials.⁹ Furthermore, a given cell/tissue test system will not necessarily possess all the possible targets on which a test chemical may act. Therefore, since each assay utilizes a unique exposure protocol or series of end points, assay batteries may be employed to address a wider range of test chemical effects.

Precise control of test article exposure dose and duration in the test system is a hallmark of *in vitro* systems. Modeling of complex tissue exposure may require specific equipment (e.g., perfusion devices) or test systems (e.g., skin models with barrier function). Prediction of the degree of toxicity to an *in vivo* tissue may require the careful modeling of the *in vivo* exposure conditions when designing *in vitro* systems. However, the *in vivo* exposure conditions are often poorly understood and potentially highly variable.^{10–12} Thus, the experimental design of the *in vitro* assay may only model a “best approximation” of the range of *in vivo* exposures. Perhaps the “easiest” exposures to model are the topical exposures of generally external tissue epithelia, such as epidermal, corneal, oral, mucosal, and genital/vaginal tissues. The modeled exposure events may be accidental exposures, such as splashing events in an industrial setting, or targeted exposures such as the application of personal care products to the skin. In general, one can make relatively simple assumptions of the dose concentrations, since the exposures frequently are of undiluted material. The modeling of planned exposures may appear relatively simple (e.g., the directed application of a personal care product to skin), since the end user is instructed on the amount and rate of product to apply. However, even under these “controlled” conditions the exposures may vary greatly—for example, the concentration of shampoo that invariably gets into a subject's eyes during normal use can vary multifold. Thus, the toxicologist may design standardized exposures in the *in vitro* test system to address specific exposure scenarios. Once established, this assay design is uniformly applied to a range of test articles of similar chemical/product class.

Because the target cells/tissues are treated in isolation, the collateral changes that might be seen in the surrounding tissues *in vivo* will not be available to demonstrate the impact of the test article on the test system. For example, in the absence of a vascular system, edema and erythema end points will be meaningless in an *in vitro* skin model. Test chemical impact on cell culture systems may be manifested as cessation of cell replication, cell death, release of inflammatory mediators, or loss of differentiated cell function. By recognizing that some changes occurring in the *in vitro* system are known to initiate collateral responses *in vivo* (e.g., release of inflammatory mediators induce erythema), some collateral changes may be readily predicted.¹³ Damage to isolated tissue systems may be assessed by similar measures as well as loss of epithelial barrier integrity and other measures of tissue degeneration.¹⁴ The mode of action of a given chemical/physical class of test articles may involve only a subset of these cellular changes *in vivo*. Determining the relevance of any of these changes to the action *in vivo* is part of the validation process.

By necessity, assessment of cell/tissue damage is generally made indirectly (e.g., by measuring an end point associated with normal cell function) and quantitatively on the treated population. Although cell death in cell culture may be detected by microscopic evaluation, such a process would be tedious and highly subjective. Understanding the impact on the population of treated cells requires methods that measure some aspects of normal cell function. The function may be continued cell replication (e.g., DNA synthesis, increased total cell number), cellular metabolic process (e.g., continued ATP synthesis, energy-dependent redox cycles), membrane integrity (e.g., loss of cytoplasmic contents), or some measure of differentiated cell function appropriate to the population under study. Isotope incorporation, vital dye uptake/metabolic conversion, and ELISA assays may be used to measure these functions on the populations. Even in the absence of functional changes in cell vitality, integrity, or protein expression/release, measuring changes in gene expression in treated tissues can provide upstream evidence of potential toxicological effects. These end points lend themselves to objective scoring by fluorometric, spectrophotometric, or other

instrument methods, which helps speed the process and reduce the subjectivity of the assessment.

As the use of *in vitro* methods in predictive toxicology has grown, there is concurrent need to demonstrate the reproducibility and relevance (to the *in vivo* response) of the assay for its intended purpose. New assays go through a process of prevalidation,¹⁵ where the transferability of the technology is demonstrated, the study protocol is finalized, and the prediction model is developed. The prediction model is used to translate the data from the *in vitro* assay into terms that can be directly compared with the *in vivo* assay/response of interest. Validation of the assay then involves testing the appropriateness of the study protocol and prediction model across several laboratories with coded samples.^{16,17} Good Laboratory Practices guidelines should be followed during this process to assure data integrity.¹⁸ Mechanisms are now in place for the formal regulatory review of new methods in Europe,¹⁹ the United States,²⁰ Japan,²¹ and Korea,²² and several *in vitro* methods have now been formally validated as stand-alone assays for classification and labeling of chemicals for ocular and dermal irritation and corrosion.^{23–28}

TABLE 5.1
Considerations in Selecting *In Vitro* Assays to Support Product Development Programs

Factors to Be Considered	Impact of Those Factors
Chemical Class of the Test Materials	
Alcohols	Not all <i>in vitro</i> systems have been characterized for their performance with a variety of chemical classes.
Organics	Carefully investigate individual <i>in vitro</i> systems to determine with which chemical classes they are compatible.
Preservatives	
Surfactants	
Acids/alkalis	
Physical Characteristics of the Test Material	
Solid/liquid	Solid or water insoluble materials should generally not be tested with a monolayer cell culture system since the test materials may not reach the target cells. Topical application assays are preferred for such materials.
Water-soluble/insoluble	Highly reactive materials may bind to the constituents of the tissue culture medium and thus be unavailable to the target tissue. Dilution into buffered culture medium will reduce extremes of pH.
Extremes of pH	Although <i>in vitro</i> tests generally require far less test material than do animal tests, there is still a considerable range of requirement among the <i>in vitro</i> systems. Some have been designed to use microquantities of materials.
Highly reactive amount of material available	
Stage in Product Development	
Single (perhaps active) ingredient	Biological activity of single ingredients may be assessed in a variety of systems including <i>ex vivo</i> tissues, tissue constructs, and monolayer culture systems. Water solubility and the end point(s) of interest will guide selection.
Mixtures	Depending on the target tissue, testing of mixtures and final formulations may require that the test system be able to model the expected exposure kinetics of the formulation as a whole. For example, dermal irritation studies may require a test system with a functional stratum corneum such as a tissue construct. In contrast, ocular irritation studies of surfactant formulations might well use <i>ex vivo</i> tissues, tissue constructs, and monolayer culture systems.
Final formulation	
Expected Level of Toxicity	
Low	The dynamic range of response of the <i>in vitro</i> system should match the expected level of toxicity of the test material. Some <i>in vitro</i> systems are designed to differentiate between weakly reactive materials; more robust systems may be useful for highly toxic materials.
Medium	
High	

(continued)

TABLE 5.1 (continued)
Considerations in Selecting *In Vitro* Assays to Support Product Development Programs

Factors to Be Considered	Impact of Those Factors
Expected Exposure to the Tissue of Interest	
Incidental/accidental Short-term or infrequent Leave-on vs. wash-off application Chronic application Use population (infants, adult, aged)	The exposure kinetics of the <i>in vitro</i> system can often be varied, and should closely match the expected <i>in vivo</i> exposures if an accurate estimate of toxicity is to be obtained.
Resolution Required of the Test	
Differentiate among similar test formulations Separating highly toxic from non-toxic materials	It may require a more sophisticated <i>in vitro</i> system to differentiate closely related materials than it would to place test materials into general classifications.
Intended Use of the Data	
Safety/efficacy screen Product development Formula optimization Claims support	The purpose of the testing should be matched to the test system; example, a simple, inexpensive <i>in vitro</i> model may be sufficient for use in screening, whereas a more sophisticated model might be necessary to characterize the effects of minor formulation changes.
Resources Available	
Funding Time Number of materials to be tested	Many <i>in vitro</i> tests can seem expensive if applied to a single test article, but are designed for easy batching of materials, which results in significant cost benefits.

TABLE 5.2
Measures of Normal Cell Function Often Used in *In Vitro* Toxicology Assays

Normal Cell Function	Assay End Points	Examples
Intact Cytoplasmic Membrane		
Permeability barrier	Exclusion of select dyes Retention of enzymatic dye cleavage products Release of cytoplasmic contents	Trypan blue, ²⁹ propidium iodide, ethidium bromide ³⁰ Calcein-AM ³¹ Lactate dehydrogenase release ⁵¹ Cr release ³²
Ion pumps and ion gradients	Changes in membrane potential Ion-specific dyes (e.g., cytoplasmic Ca ²⁺ gradient)	Patch-clamp membrane potentials ³³ Fluo3-AM ³¹
Membrane lipid integrity	Release of arachidonic acid metabolites (eicosanoids and leukotrienes)	ELISA-PGE ₂ ³⁴
Cellular Metabolism (continued regeneration of ATP/NADH)		
Production of CO ₂ /lactate	Rate of release of acidic metabolic by-products into the medium	Cytosensor TM microphysiometer ^{35,36}
Cellular ATP concentration	Concentration of ATP and/or ATP/ADP ratio in the population	Luciferin/luciferase assays ³⁷
Renewal of NADH/NADPH (cell/population redox potential)	Vital dye reduction (e.g., tetrazolium dyes)	MTT, XTT, alamarBlue ^{®38,39} Calcein-AM
Maintenance of energy-dependent ion gradients	Assessment of the lysosomal H ⁺ ion gradient Mitochondrial H ⁺ ion gradients	Neutral red uptake ^{40,41} Rhodamine 123 ⁴²
Maintenance of ATP-dependent polymerized cytoskeletal proteins	Staining of filamentous actin in the corneal stromal keratocytes	Phalloidin ⁴³

TABLE 5.2 (continued)
Measures of Normal Cell Function Often Used in *In Vitro* Toxicology Assays

Normal Cell Function	Assay End Points	Examples
	Cell Replication	
Increase in cell number	Cell counts over time Formation of cell colonies Increases in total protein	Electronic cell counters Clonogenic assays ⁴⁴ Kenacid blue or Coomassie blue ⁴⁵
Scheduled (S-phase) DNA synthesis	Increases in vital dye uptake/reduction Incorporation of nucleotides S-phase specific proteins	Neutral red, Tetrazolium dyes ³ H-thymidine ^{46,47} Bromodeoxyuridine ELISA, immunofluorescence, or fluorescent-activated cell sorter analysis
	Maintenance or Induction of Differentiated Function	
Normal cytoskeleton	Cell shape	Microscopic observation, immunofluorescence
Production of “marker” proteins	Constitute expression of appropriate membrane markers Induction/release of protein products (e.g., collagen from fibroblasts)	ELISA, immunofluorescence, or flow cytometry and fluorescent-activated cell sorter analyses ELISA

TABLE 5.3
Examples of Vital Dyes Used to Assess Cell Viability

Cell Function	Dye	Detection	Application	Notes
Membrane integrity	Trypan blue ⁴⁸	Light microscopy (visible light)	Rapid screen for dead cells used in conjunction with manual cell counts.	Will eventually penetrate cells if the incubation extends over 30 min.
	Calcein-AM ³¹	Fluorescence: Excitation/emission 485–495/520–530 nm	Readily penetrates viable cells where AM is cleaved by esterases. Resulting dye is fluorescent and is retained by the intact cell.	Low background fluorescence from Calcein-AM.
	Ethidium bromide ³⁰	Fluorescence: Excitation/emission 510–520/595–605 nm	Stain DNA but do not pass through the intact cell membrane. Marker of dead cells. Often used in combination with Calcein-AM in “live/dead” stain combinations.	Generally not used alone since only dead cells are stained. Cells that have completely degenerated are lost from the count.
	Propidium iodide ³⁰	Fluorescence: Excitation/emission 530–540/620–640 nm	Stain DNA but do not pass through the intact cell membrane. Marker of dead cells. Often used in combination with Calcein-AM in “live/dead” stain combinations.	Generally not used alone since only dead cells are stained. Cells that have completely degenerated are lost from the count.
Cellular redox potential ^{38,49,50}	MTT 3(4,5dimethylthiazol2yl)- 2,5-dephenyl tetrazolium bromide	Spectrophotometry: OD at 540–570 nm	MTT is converted from the oxidized form to the reduced form by the NADH ⁺ dependent reaction catalyzed by cytoplasmic and microsomal associated enzymes. Upon reduction, dye turns from a yellow moderately water-soluble form to a blue/black insoluble form. The dye is then extracted from the cells or tissues and quantitated.	Little interference from oxidized dye. Good choice where nonspecific binding is expected (e.g., tissue constructs). Increased glycolytic activity will increase tetrazolium dye reduction. If present, reducing agents will reduce the dye and give a false indication of viability. Before testing, screen-test chemicals for dye reduction if they will contact the dye.

(continued)

TABLE 5.3 (continued)
Examples of Vital Dyes Used to Assess Cell Viability

Cell Function	Dye	Detection	Application	Notes
	XTT sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide	Spectrophotometry: OD at 540–570 nm	XTT is converted from the oxidized form to the reduced form by the NADH ⁺ dependent reaction catalyzed by cytoplasmic-associated enzymes. Reduced XTT is water-soluble, so extraction is not needed.	Little interference from oxidized dye. Good choice where nonspecific binding is expected (e.g., tissue constructs). Increased glycolytic activity will increase tetrazolium dye reduction. If present, reducing agents will reduce the dye and give a false indication of viability. Before testing, screen-test chemicals for dye reduction if they will contact the dye.
	WST-1	Spectrophotometric: OD at 540–570 nm	Dye is converted from the oxidized form to the reduced form by the NADH ⁺ dependent reaction catalyzed by plasma membrane-associated enzymes. Uses intermediate electron acceptor to pass through the plasma membrane. The reduced dye is water-soluble; so no extraction is needed.	Little interference from oxidized dye. Good choice where nonspecific binding is expected (e.g., tissue constructs). Increased glycolytic activity will increase tetrazolium dye reduction. If present, reducing agents will reduce the dye and give a false indication of viability. Before testing, screen test chemicals for dye reduction if they will contact the dye.
	Alamarblue®	Fluorescence: Excitation/emission 530/590 nm	alamarBlue is converted from the oxidized form to the reduced form by the NADH ⁺ -dependent reaction catalyzed by cytoplasmic/membrane-associated enzymes. The reduced dye is water-soluble; so extraction is needed.	Little interference from oxidized dye. Increased glycolytic activity will increase tetrazolium dye reduction. If present, reducing agents will reduce the dye and give a false indication of viability. Before testing, screen-test chemicals for dye reduction if they will contact the dye. Use with caution with tissue constructs since overprediction of tissue viability has been reported.
Energy-dependent ion gradients				
Lysosomal pH gradient ^{3,51,52}	Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride)	Spectrophotometric: OD at 540–550 nm	At physiological pH, the uncharged dye passes through membranes. The dye develops a net charge at the lower pH of the lysosome and so does not readily pass out of the lysosome. The proton gradient across the lysosomal membrane is energy-dependent. Dye must be extracted for spectrophotometric analysis.	Loss of this pH gradient, either through mortality/morbidity of the cell or permeation of the membrane, will release retained neutral red or prevent its accumulation. Nonspecific dye binding to test materials or tissue matrix will interfere with the assay. Not all cell types have sufficient lysosomal content for a strong signal.
Ca ²⁺ gradients	Fluo-3 AM ³¹	Fluorescence: Excitation/emission 485/500–530 nm	Enters the cell where cleavage of the AM enhances retention. Fluorescence proportional to free Ca ²⁺ bound to the dye.	Changes may be rapid and may require Ca ²⁺ in the extracellular medium.
Energy-dependent esterases	Calcein-AM ³¹	Fluorescence: Excitation/emission 485–495/520–530 nm	Readily penetrates the viable cell where the AM cleavage by esterases is energy-dependent. Resulting dye is fluorescent and is retained by the intact cell.	Low background fluorescence from Calcein-AM.

TABLE 5.4
Factors in the Design of Cell-Based Assays

Factors to be Considered	Impact of those Factors
Selection of the Target Cells	
Species	Cells from the desired species and target tissue may not be available. A surrogate cell type or species may be sufficient. Normal cells may be required when differentiated function is assessed but may have a short life span in culture. Transformed/immortalized cells allow further characterization and more potential for genetic manipulation. A differentiated, often nondividing, cell type may be required to provide the normal xenobiotic biotransformation capabilities or desired cellular targets for the test chemical.
Tissue/organ of interest	
Degree of differentiation required	
Normal	
Transformed/immortalized	
Cell Preparation and Maintenance	
The goal is to achieve a consistent starting population with the desired characteristics.	Procedures for maintaining stock cells include medium selection, initial seeding and refeeding, time/cell density for reseeding (passaging), and removal of the cells from the growth substrate.
Testing for adventitious agents	Cells should be free of bacteria (including mycoplasma), fungi, and other contaminants.
Selection of the Appropriate Substrate	
Plastic	Most nontransformed cells are anchorage-dependent. Cell adherence, polarization, and differentiation may be modulated by the substrate selected. Certain transformed or lymphocytic cell lines may grow well in suspension but can be difficult to manipulate in multiwell plates. Special techniques are needed to change the medium without losing cells. Notable improvements in maintaining/enhancing primary cell function and states of differentiation can be achieved using gelatin and collagen matrices (e.g., Matrigel). Complex scaffolding and culture techniques are used for maintaining or creating 3D tissues.
Protein/collagen substrates	
Semipermeable support	
Suspension	
Culture and Treatment Medium	
Serum-containing	Serum may bind the test material altering the effective exposure to the target cells. Serum may also complicate analysis of cell products. However, only a small fraction of available cell lines are adapted to serum-free medium.
Serum-free	
Defined	
Culture Format	
Dishes (treated petri dishes)	Dishes and flasks are traditional vessels for maintaining stock cultures but may be cumbersome for assays with large numbers of replicate cultures. Multiwell plates are used to provide the required number of replicate cultures and facilitate handling and end point determination. When selecting a multiwell plate format, the required well surface/volume may be dictated by the assay end points planned. When high throughput (HTP) multiwell plate formats are used (e.g., 1536 well plates), cell morphology and function may be impacted, and thus should be screened for impact upon assay performance.
Flasks	
Multiwell plates	
Starting Cell Density	
Dividing	Cell density should be high enough to provide strong end point signals. Densities of dividing cells should allow controls to remain in log-phase growth throughout the assay. Nondividing/differentiated cells (e.g., normal hepatocytes) may require high seeding densities to maintain their differentiated state.
Nondividing cells	
Assay Dose Range and Test System Exposure	
Full range of doses to cover expected range of toxicity	Dose steps (e.g., half or quarter log dilution intervals) should be appropriately spaced to provide resolution among similar test materials, and the steps should be similar for all materials being compared. The volume of treatment medium as well as the test material concentration may impact the effective dose to the cells due to partitioning of lipophilic materials into the cell membrane or other tissue structures.
The duration of exposure	Exposure period may be modeled after expected <i>in vivo</i> exposure. Breakdown of the test material may reduce the effective exposure period. Exposure time may also be dictated by the end point of interest (e.g., the evaluation of chemicals affecting cytokinesis may require an exposure time equal to at least one cell cycle <i>in vitro</i>).
“Expression” period before the end point assay(s) are performed	Manifestations of the action on the cells may not be immediate (e.g., antimetabolites) requiring hours or days before differences are observed.
Examples of End Points in a Cytotoxicity Assay: Measuring Cell Death	
Measurements of the metabolic state	ATP concentration Neutral red–lysosomal sequestering Tetrazolium or alamar blue dye reduction Calcein-AM cleavage Direct measurement (cytosensor microphysiometer)
Measurements of membrane integrity	Trypan blue, calcein-AM, or propidium iodide uptake “Live/dead” nuclear stains Cytoplasmic enzyme release (LDH)

TABLE 5.5
Roles of the Negative Control, Positive Control, and Benchmark Materials

Negative control
<ul style="list-style-type: none">• Provides the baseline values against which the test article and positive control treated cultures are compared• Generates absolute values for the viability end point (i.e., neutral red or MTT) in the tissue that would be part of the acceptance criteria for the assay; these values would be expected to reflect normal constitution (e.g., number of cells) and function (e.g., metabolic state) of the cells or tissue• Provides a baseline for treated but undamaged tissue for functional measures (i.e., BCOP, CEET)
Positive control
<ul style="list-style-type: none">• Ensures the integrity of the test system and proper execution of the assay• Needs to be included each time the assay is performed• Provides one of the acceptance criteria for the assay (based on historical values)• Its use (concentration, exposure time, etc.) is consistent for each assay so that a historical range of responses can be generated. The response to the positive control in each assay trial is compared with the historical control range to determine that the trial is acceptable. It is not dependent upon the type of unknown material being tested in the assay• Should allow detection of over- and under-response in an assay relative to its historical performance; positive controls that give only extreme responses are of little use• Lot release testing by tissue construct manufacturers established the acceptability of the test system at the point of manufacture but is not a substitute for concurrent positive control tested in parallel with the unknown articles in the final assay
Lot release criteria
<ul style="list-style-type: none">• Lot release testing is an essential feature of commercial tissue models. It should measure the essential performance characteristics of the tissue. For example, a skin model should be tested to show the integrity of the stratum corneum and the robustness of the tissue viability signal (e.g., the number of viable cells contributing to the viability signal). While not every end point may be tested, the lot release criteria should give the user confidence that the tissue is performing correctly for the assay(s) to be performed and should be able to quantitatively show hyper- and hyposensitivity against established historical values
Benchmark(s)/reference materials
<ul style="list-style-type: none">• Selected to match to the chemical/product type of the unknown(s) being tested• Used to set upper and/or lower limits of response against which the unknown(s) are judged• Should have an extensive <i>in vivo</i> database (e.g., clinical or other reference assay)• Will change in an assay depending on the types of classes of materials being tested

Source: Harbell, J. W., Southee, J. A., and Curren, R., The path to regulatory acceptance of *in vitro* methods is paved with the strictest standards. In *Animal Alternatives, Welfare and Ethics*, van Zutphen, L.F.M. and Balls, M. Eds., Elsevier Science, Amsterdam, The Netherlands, 1997, pp. 1177–1181.

TABLE 5.6
Laboratory Safety Requirements for the Use of Human Cells

Precautions in a tissue culture laboratory using human tissue focus on the following:
<ul style="list-style-type: none">• Concerns with human pathogens such as Hepatitis B, HIV, and other potentially unrecognized viruses• Universal precautions and other legal requirements, as outlined by the Centers for Disease Control, which must be observed• Documented sources of cells or tissues; certificate of test results should be available if commercial sources used• Performing work in a biological safety cabinet (vertical laminar flow hood) to protect the operator and preserve the sterility of the test system• Regular disinfection of surfaces to reduce contamination• Sterilization of spent medium, plasticware, and cell cultures before disposal

Source: CDC, *Biosafety in Microbiological and Biomedical Laboratories*, U.S. Government Printing Office, Washington, DC, 1993.

ASSESSMENT OF OCULAR IRRITATION POTENTIAL

Prediction of ocular irritation potential requires modeling both a target tissue and relevant exposure kinetics. This modeling is not necessarily a duplication of the *in vivo* condition but should be appropriate for the physicochemical class of the test material and potential modes of action on the tissue targets. Physicochemical properties, such as water solubility, impact the effective amount and duration of the exposure to the tissue. The test system must address the expected mode(s) of action, both in the end point(s) measured and the time course for that end point change to develop. For example, water-soluble surfactant materials might be expected to act rapidly on the cell membranes to produce cell lysis. Nonsurfactant preservatives might target cellular metabolic or replication functions and so require much more time to manifest their impact. Water-insoluble material may never reach the cells in a submerged test system. These materials would require direct application to the tissue to model their *in vivo* exposures.

This section reviews a range of *in vitro* assays used to predict ocular irritation and discuss the selection process based on various applications. Assays may be divided into classes according

to their exposure conditions, exposure kinetics, time to end point determination, and the end points themselves. For example, the Cytosensor microphysiometer, neutral red release, short time exposure (STE), and red blood cell lysis assays are all based on short exposures to serial dilutions of the test article and immediate assessment of the cellular change (membrane damage). To this end, they are generally used to test surfactant materials as are the fluorescein leakage assays. The *ex vivo* models (bovine cornea and enucleated eye models) and 3D-engineered human tissue constructs allow direct application of the test material to the tissue, but differ considerably in the end points measured. These two types of models can be quite complementary in that they address different degrees of toxicity and end points. The human tissue constructs are able to resolve well in the very mild-to-moderate irritation range, while the *ex vivo* tissues can be used to address mild/moderate to severe materials. Used together, they can provide measures of cell death, corneal opacity, epithelial cell loss, and overall histological changes.

Scott et al. have suggested four broad modes of action that account for chemical irritation/corrosion to the eye.⁹ These include membrane lysis characterized by rapid, progressive loss of cell layers in the exposed tissues through contact with surfactants, organic solvents, and other membrane-active chemicals. Protein coagulation/denaturation also leads to rapid loss of cell viability and attacks the stromal elements of the ocular tissues. Acids, cationic surfactants, and some organic solvents can induce this kind of damage. Saponification of tissue lipids by exposure to alkali materials leads to progressive, penetrating injury as the alkali moves deeper into the tissue. Finally, damage to macromolecules (i.e., DNA alkylation, mitochondrial injury) can lead to a delayed pattern of injury because the cells show delayed necrotic or apoptotic cell death. This type of injury is characteristic from exposure to peroxides, mustards, or oxidizers. The delayed type of injury can be more difficult to detect *in vitro* if the assay does not allow for the expression of delayed injury.

The ultimate goal of the *in vitro* or *in vivo* assays for eye irritation is the prediction of the degree and duration of the ocular injury that the test material could induce. For exposures *in vivo*, Jester and Maurer have shown that the initial depth of injury in the cornea is highly predictive of the degree and duration of that injury.^{13,55–57} In a series of studies using chemicals that act across the modes of action described earlier, they found that the depth of injury (cell death) was the critical determinant. They used the depth of cell death 3 h after treatment (except for bleaches and peroxides where a slightly longer postexposure period was required⁵⁸) to predict recovery or the lack of recovery. Those chemicals that killed cells in the epithelium only, induced clearly reversible injury (slight to mild). Those chemicals that killed cells in the upper part of the stroma (very close to Bowman's membrane) induced moderate injury that would recover in 21 days. Those chemicals that induced injury deeper into the stroma induced severe to corrosive injury. These observations are critical for the development and selection of *in vitro* assays for eye irritation. Thus far, it has been difficult for one assay to carefully resolve depth of injury from minor damage to the epithelial surface on one

hand and killing of the keratocytes in the deeper stroma on the other. Therefore, batteries of tests may be employed depending on the particular need. As proposed by Scott et al., one might start with an assay focused on the epithelium (minimal to mild irritation potential) if the test material is expected to be slight to mild. This is the common approach for the cosmetics industry. On the other hand, certain chemicals or product classes are expected to be in the moderately irritating class, and the concern is to identify those members that are in the severe category. Then an assay that is designed to resolve in that range would be employed. These test systems allow one to measure the depth of injury into the stroma directly or project such depth of injury indirectly.

The isolated cornea test systems are often employed for resolving irritation potential in the moderate to severe range.²³ Certain modes of action by the test chemicals on the cornea can be more readily resolved by the standard end points of the assays (opacity, swelling, fluorescein permeability/staining), but for more critical assessment and to address the delayed types of cell death, histology has been performed on the corneas fixed at the end of the assay.^{59,60} Depth of injury is determined by scoring cell loss/killing in each of the three corneal layers; epithelium, stroma, and endothelium. Epithelial lesions include loss of layers of cells (often progressively from the squamous cells, through the wing cells, and then through the basal cells), precipitation/coagulation of cellular components or "stripping" of cellular components (leaving a meshwork of cellular residue). Depth of injury in the epithelium is relatively easy to estimate. With loss of epithelial barrier integrity, water moves into the stroma leading to stroma swelling manifested by increased stroma thickness and separation of the collagen bundles. Probably, the more critical end point is the action on the keratocytes. The depth of keratocyte injury is used to determine moderate-to-severe irritants. Endothelial loss is associated with severe irritation/corrosion. It is critical to compare the test material-treated corneal sections with negative control-treated corneal sections tested and processed in parallel to differentiate between test material-induced changes and artifacts of handling or staining.

Histological evaluation of corneas treated *in vivo* is facilitated by the inflammatory changes (influx of inflammatory cells) to mark the zone of cell death. *In vitro*, there is no influx of cells and so depth of injury is determined by estimating the viability of keratocytes, through the depth of the stroma, based on cell morphology and nuclear staining. Given the importance of this measurement, a more objective method to define the zone of cell killing would be desirable. Jester has used the presence of filamentous (polymerized) actin in the keratocyte cytoplasm as an indicator that the cell was alive (making ATP) at the time of fixation.⁴³ The corneal sections can be stained with rhodamine-conjugated phalloidin to make live cells and establish the depth of cell killing in the stroma.⁶¹

Several of the regulatory schemes for grading eye irritation potential *in vivo* use time to recovery as a primary driver of classification. To that end, some efforts have been

made to measure recovery with *in vitro* test systems,^{62,63} where the end point is the re-establishment of a corneal epithelial layer. These have proven particularly challenging. Recovery, *in vivo*, involves much more than simple re-epithelialization of the cornea. Essential to the process is the cessation of stromal inflammation and restoration of the keratocyte network. This process has proven difficult to measure, quantitatively, in the current models and end points. A major part of the challenge is to maintain

the *ex vivo* test system for the extended culture periods required. Swelling and degradation of the stromal elements have been difficult to control. Additionally, the current *ex vivo* recovery models do not address the inflammatory process that occurs *in vivo*, or its impact upon corneal recovery downstream. Fortunately, the initial depth of injury model makes clear predictions for recovery/nonrecovery without the need to maintain the test systems for extended periods (Tables 5.7 through 5.19).

TABLE 5.7
Characteristics of Common *In Vitro* Assays for Ocular Irritation

Designation	General Description	Method of Applying Test Material
Bovine corneal opacity and permeability (BCOP) ^{64,65}	Living bovine corneas treated with test material and changes in opacity and permeability are measured by instrument.	Test materials applied neat or at end-use concentrations directly to the epithelial surface of the cornea.
Chorioallantoic membrane vascular assay (CAMVA) ^{66,67}	Chorioallantoic membrane of a fertilized chicken egg treated and scored for vascular constriction, hyperemia, and hemorrhage.	Generally increasing dilutions of test material applied to the membrane of multiple eggs and damage to the membranes recorded; the dose effective in 50% of the eggs is calculated.
Cytosensor microphysiometer ^{35,36}	Cells held in a flow-through chamber with a sensor chip which measures pH; cells treated and changes in cellular metabolism (release of H ⁺ ions) recorded in real time.	Generally increasing concentrations of test material added to growth medium until a predetermined end point (decrease in metabolism) is reached.
Enucleated chicken or rabbit eye ^{24,68}	Isolated eyes treated topically and subsequently scored for opacity, corneal swelling, and fluorescein staining.	Test materials applied neat or at end-use concentrations directly to the cornea.
Fluorescein leakage ^{69–71}	Target cells (primary or continuous) capable of forming tight junctions are grown in submerged monolayer or multilayered culture; medium may be removed for dosing.	Either increasing dilutions of test material added to growth medium or cell surface for a set time, or a single concentration is added for varying times; end point is induction of permeability of the tissue to fluorescein.
Hen's egg test chorioallantoic membrane (HETCAM) ^{72,73}	Chorioallantoic membrane of a chicken egg treated and scored for protein coagulation, hyperemia, and hemorrhage.	Test materials applied neat or at in-use concentrations directly to the membrane and damage to the membrane recorded.
IRRITECTION ^{TM2}	Precipitation/turbidity of protein in a nonviable commercially supplied matrix is the end point; meant to mimic opacity formation in the cornea.	Either dilutions or neat test material added to a membrane bullet over a responding protein matrix.
Neutral red release ⁷⁴ Neutral red uptake ³	Target cells (primary or continuous; fibroblasts or epithelial-like) grown in submerged monolayer culture in multiwell plates; exposures may be minutes (release) or days (uptake).	A range of test article dilutions prepared in growth medium to reach a predetermined end point (generally cytotoxicity).
Red blood cell ⁷⁵	Red blood cells exposed to test material.	Dilutions of test material used; lysis (release of hemoglobin) and hemoglobin denaturation monitored.
STE test ⁷⁶	Target cells (SIRC cells, a continuous cell line derived from rabbit cornea) grown in submerged culture at clonal densities and scored for reduction in cell viability.	A series of fixed test article dilutions prepared in saline (or comparable vehicle) to reach a predetermined end point (cytotoxicity) at each dilution.
3D engineered human tissue construct assays ^{6,77–79}	3D human-reconstructed tissue grown with top surface exposed to air; cell viability and inflammatory mediator release are common assay end points.	Test materials applied neat or at end-use concentrations directly to the tissue construct and cell killing measured.

TABLE 5.8
Assay Selection Considerations for *In Vitro* Ocular Irritation Testing: Impact of Water Solubility

Assay system compatible with water-soluble materials

- BCOP assay
- 3D engineered human tissue construct assays
- Fluorescein leakage assays
- Submerged cell-based systems
 - Neutral red uptake/release assays
 - Cytosensor microphysiometer
 - STE test
 - Red blood cell lysis
- Chorioallantoic membrane (CAM)-based systems
- IRRITECTION™

Assay system applied to hydrophobic formulations

- BCOP assays
- 3D engineered human tissue construct assays
- Fluorescein leakage assays (limited)
- CAM-based systems
- IRRITECTION™
- STE test (limited using lipophilic vehicle)

Assay system suitable for undiluted ingredients/formulations

- BCOP assays
- 3D engineered human tissue construct assays
- HETCAM
- IRRITECTION™

TABLE 5.9
Advantages and Disadvantages of Dilution-Based Assays^a for Ocular Irritation

Advantages	Disadvantages
Rapid to execute using multiwell plate formats.	Cannot be used easily with water-insoluble materials.
Most are machine scored based on dye incorporation/reduction.	Dilution effects may mask toxicity of neat material (e.g., alcohols).
Generally very cost-effective—Multiple materials may be tested concurrently.	Change in the physical form, example, solids to solutions, may impact exposure kinetics.
Seem to work well with surfactants and surfactant-based formulations.	Buffering effects of the medium may affect toxicity significantly
Often differentiate well between very mild materials.	Possible reaction of the test material with the solvent/medium components.

^a Assays in which serial dilutions of the test material are applied to the test system, and the end point is the concentration of test material that causes a selected response.

TABLE 5.10
Advantages and Disadvantages of Topical Application Assays^a for Ocular Irritation

Advantages	Disadvantages
• Material is tested in its “native” form, that is, in the same form as an <i>in vivo</i> exposure	• Test substrate can often be expensive
• Exposure of the target tissue can be assured	• Exposure times may be inconveniently long, requiring work past the normal workday
• In some models, exposure time can be selected to match expected <i>in vivo</i> exposure	• Solid materials may require special handling to apply to the test system
• Exposure measured through the depth of the tissue	• Availability of tissue in some markets
	• Limited shelf life

^a Assays in which only the neat or end-use concentration of test material is applied to the test system (i.e., *ex vivo* or reconstructed tissue), and the end point(s) depend on the dynamic range of the test system and/or exposure time vs. end point activity.

TABLE 5.11
Further Characterization of Common *In Vitro* Assays for Ocular Irritation

Test	End Point	Resources Needed	Skill Level
BCOP ^{64,65}	Opacity, permeability, opacity and permeability, histology	Specialized equipment (opacity), spectrophotometer	General laboratory skills; histology, if included
CAMVA ^{66,80}	Vascular hemorrhage, constriction, and dilation	General laboratory equipment, shell-cutting tool	General laboratory skills with training for scoring lesions
Cytosensor microphysiometer ^{35,36}	Cellular metabolism	General tissue culture laboratory equipment, Cytosensor (expensive)	Tissue culture skills
Enucleated chicken/rabbit eye ^{24,68}	Opacity, corneal swelling, fluorescein staining, histology	Specialized equipment (expensive)	General laboratory skills
Fluorescein leakage ^{69–71}	Increased epithelial permeability	General tissue culture laboratory equipment, spectrophotometer	Tissue culture skills
HETCAM ^{72,73}	Vascular damage, coagulation	General laboratory equipment	General laboratory skills with training for scoring lesions
IRRITECTION ^{TM2}	Precipitation	Specialized equipment, multiwell plate reader	General laboratory skills
Neutral red release ⁷⁴	Cytotoxicity/membrane damage	General tissue culture laboratory equipment, 96-well plate reader	Tissue culture skills
Neutral red uptake ³	Cytotoxicity	General tissue culture laboratory equipment, 96-well plate reader	Tissue culture skills
Red blood cell ⁷⁵	Membrane lysis	General laboratory equipment, spectrophotometer	General laboratory skills
STE test ⁷⁶	Cytotoxicity	General tissue culture laboratory equipment, 96-well plate reader	Tissue culture skills
3D engineered human tissue construct assays ^{6,77,78,79}	Cytotoxicity	General tissue culture laboratory equipment, spectrophotometer/96-well plate reader	Some tissue culture skills

TABLE 5.12
Sample Protocol Outline for the STE Test

Theory

The potential eye irritancy of a test chemical is determined in this dilution-based cell monolayer assay by measuring the relative viability of Statens Serum Institut Rabbit Cornea (SIRC) cells after a 5-min test chemical exposure. Relative cell viability is determined by comparing the cellular reduction of MTT in treated and control cultures. A rank classification (minimal, moderate, or severe) of eye irritation potential is determined by testing two dilutions of 5% and 0.05% of test chemical. The STE assay results from these dilutions correlated well with Draize 100% and 10% doses.⁷⁶

Applications and Use

- Testing of neat formulations is not modeled, since test chemicals are applied as dilutions. However, test chemicals may be diluted in HBSS, 5% DMSO/95% saline, or mineral oil.
- The test system uses serum-free vehicles, which eliminate the possibility of serum protein: test chemical interactions.
- 5-min exposure time closely models likely accidental exposures.
- Validation studies showed good correlation with Draize scores, EU classification, and GHS labeling for eye irritation potential.^{82,83}

Experimental Procedure

Target Cell Preparation

- Cell cultures are maintained at 37°C ± 1°C in a humidified atmosphere containing 5% ± 1% CO₂ (standard culture conditions).
- Cells are subcultured when the stock culture is 50%–80% confluent.
- Cells are maintained in SIRC growth medium: (Eagle's minimal essential medium [EMEM]) supplemented to contain 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, and 100 U/mL/100 µg/mL penicillin/streptomycin.
- Cells are used 3 weeks to 3 months after reconstitution (or up to passage +25).

TABLE 5.12 (continued)**Sample Protocol Outline for the STE Test**

- A cell suspension is prepared and seeded into a 96-well plate (3.0×10^3 cells/well) for a 120-h incubation under standard culture conditions.
- On the day of dosing, cell monolayer should be $\geq 80\%$ confluent.

Sample and Positive Control Preparation

- Test chemicals are diluted to 5% and 0.05% in physiological (0.9%) saline. If insoluble or immiscible in saline, then 5% DMSO/95% saline or mineral oil may be used.
- The positive control, 0.01% SLS, is prepared in physiological saline and tested on each 96-well plate.
- Each 96-well plate contains applicable solvent and medium controls.

Assay Procedure

- 96 h after cell seeding, the media from each well is aspirated.
- Each test chemical dilution (5% and 0.05%), the positive control, and the solvent and medium controls are dosed in triplicate wells for 5 min.
- After the 5-min treatment, the test or control treatments are aspirated in the same order in which they were dosed.
- The plates are incubated for 5 min at room temperature.
- The wells are rinsed twice with 200 μ L of PBS and the rinsate aspirated well-by-well.
- 200 μ L of 0.5 mg/mL MTT prepared in SIRC growth medium is added to all test wells and incubated at standard culture conditions for 2 h.
- After 2 h, MTT is decanted and 0.4N acidic isopropanol is added to all test wells. The plate is shaken at room temperature for at least 60 min.
- After 60 min, the absorbance of the MTT at 570 nm (OD_{570}) is measured with a 96-well plate reader.

Data Evaluation

- Cell viability is determined by comparing the mean-corrected OD_{570} of the test chemical-treated wells to the mean-corrected OD_{570} of the applicable solvent control wells.
 - Mean cell viability and standard deviation (SD) of viabilities of each tested dose are calculated for each definitive assay. Three definitive assays are performed to determine assay validity ($SD < 15\%$), STE classification (irritant or NI), and STE rank Score (mild, moderate, or severe irritation potential).
 - *STE classification*: Mean cell viability of the 5% dose is used to determine the STE classification⁸²:
 - If the mean cell viability is $>70\%$, the test chemical is predicted to be a NI.
 - If the mean cell viability is $\leq 70\%$, the test chemical is predicted to be an eye irritant.
 - *STE irritation rank*: Scores of 0, 1, or 2 are given to the 5% and 0.05% dilutions, based upon the relative viability.
 - 5% dilution: a score of 0 is given if the cell viability is $>70\%$; and a score of 1 if the cell viability is $\leq 70\%$.
 - 0.05% dilution: a score of 1 is given if the cell viability is $>70\%$; and a score of 2 if the cell viability is $\leq 70\%$.
- The sum of the scores is used to determine the STE irritation rank: Minimal (score = 1), Moderate (score = 2), or Severe irritant (score = 3).⁸²

TABLE 5.13**Sample Data from the STE Test**

Chemical	Chemical Class	GHS	STE 5% ^a	STE 0.05% ^a	STE Category	STE Rank
Benzalkonium chloride, 10%	Surfactant (cationic)	1	2.1	3.1	Irritant	Severe
Diethylethanolamine	Amine	1	0.2	91.5	Irritant	Moderate
Sodium lauryl sulfate, 15%	Surfactant (anionic)	1	0.3	-0.8	Irritant	Severe
Calcium thioglycollate	Organic salts	2A	7.0	109.8	Irritant	Moderate
2-Butanone	Ketone	2A	44.7	100.7	Irritant	Moderate
1-Octanol	Alcohol	2A	-0.5	96.8	Irritant	Moderate
Ethanol	Alcohol	2A	98.2	97.1	NI	Mild
Tween80	Surfactant (nonionic)	NI	114.1	104.6	NI	Mild

Source: Select data from Takahashi, Y. et al., *Toxicol. In Vitro*, 22, 760, 2008.

^a Relative viability (% of controls).

TABLE 5.14**Sample Protocol Outline for the BCOP Assay****Theory**

The potential ocular irritancy/toxicity of a test article as measured by the induction of either or both (1) corneal opacity, which may be caused by protein coagulation/cell precipitation, or the induction of stromal swelling; and (2) corneal permeability to fluorescein, reflecting a degradation/loss of the corneal epithelium. Histopathological changes may be used to assess depth of injury to the tissue.^{59,65,83–86}

Applications and Use

- The BCOP model is a biologically complex *ex vivo* model with end points similar to many human corneal responses, yet is relatively inexpensive.
- Histological evaluation of the tissue can be performed to measure the depth of injury.
- BCOP is particularly suited for moderate to severely aggressive materials, where other models may not be suitable.
- Multiple end points allow the investigation of mechanisms of action.

Experimental Procedure**Bovine Cornea Preparation**

- Bovine eyes are received from the abattoir shortly after the slaughter of the animal; so the cells are still viable.
- The corneas are excised, mounted on the BCOP chambers, and allowed to equilibrate in MEM supplemented with 1% FBS and without phenol red (complete MEM) for 1 h.

Sample and Positive Control Preparation

- The samples can be tested undiluted (neat) or at any concentration in a variety of solvents.
- Positive and negative controls are tested and are the bases for assay acceptance criteria and reproducibility.
- Individual positive controls are used for the testing of either liquid or solid test materials. Ethanol at ~100% is used as the positive control for the liquid protocol and Imidazole at 20% in complete MEM for the solid protocol.

Assay Procedure

- The bovine eyes are harvested shortly after the animal is slaughtered, and shipped in cold HBSS supplemented with 1% penicillin/streptomycin.
- The bovine corneas are carefully checked for any damage (opacity, cuts, etc.) before being excised and mounted on the special BCOP chambers with complete MEM and incubated at 32°C for 1 h.
- After the 1 h incubation, an initial opacity for each cornea is read in an opacitometer and recorded.
- The corneas are divided into groups between three and six corneas per test article per exposure time and exposed to the test article at the concentration requested.
- A 750 µL aliquot of test material is exposed to the cornea. The material can be tested at any concentration and over a range of exposure times.
- The test article is rinsed from the cornea and opacities read and recorded before an additional 32°C incubation.
- Posttreatment incubation times may be varied to enhance postexposure expression of irritancy.
- A postincubation opacity reading is taken on each cornea and recorded.
- The initial opacity reading is subtracted from the postincubation opacity to calculate the final opacity reading.
- A permeability test is performed to measure the passage of fluorescein stain through the cornea. A medium sample is removed from the posterior end of the chamber and measured spectrophotometrically (490 nm) to determine the amount of fluorescein leakage (OD₄₉₀).
- Both opacity and permeability scores are used to calculate the final BCOP score.
- Corneas may also be fixed, sectioned, and examined in histopathology.

Data Evaluation

- Sina et al. have proposed a scoring system integrating opacity and permeability: *in vitro* score = opacity + 15 × permeability.¹⁴
- 0–25 is considered a mild irritant, 25.1–55 is considered moderate, and 55.1 and above is considered severe.^{14,23}
- It is important to address the individual contributions of opacity and permeability relative to the chemical class tested.^{85,86}

TABLE 5.15
Sample BCOP Assay Data

Chemical	Opacity	Permeability (OD ₄₉₀)	<i>In Vitro</i> Score	Predicted Irritancy Class ⁶⁵
Nonsolvent, Nonsurfactant Liquids				
Trichloroacetic acid (30%) ^a	205.5	0.049	206.2	Severe
2,6-Dichlorobenzoyl chloride ^a	21.7	0.026	22.1	Mild
Ethyl-2-methylacetoacetate ^a	17.2	0.083	18.5	Mild
Glycerol ^a	2.7	0.099	4.2	Mild
Pyridine ^a	92.7	2.457	129.5	Severe
Polyethylene glycol 400 ^a	3.7	0.005	3.7	Mild
Solvents				
Butanol ^b	15.8	1.456	37.7	Moderate
Isopropanol ^b	33.0	0.703	43.5	Moderate
Methyl ethyl ketone ^b	72.3	0.059	73.2	Severe
Acetone ^b	144.5	1.032	160.0	Severe
n-Hexane ^a	7.7	0.030	8.1	Mild
Methyl acetate ^a	54.5	0.245	58.1	Severe
Surfactants				
Polyglyceryl-12-laurate ^b	0.3	0.005	0.4	Mild
Tween-20 (20%) ^b	1.0	0.010	1.1	Mild
Adult shampoo (10%) ^b	3.7	0.526	11.6	Moderate ^c
Sodium lauryl sulfate (10%) ^b	6.1	1.712	31.8	Severe ^c
Alkyl dimethyl benzyl ammonium chloride ^b	48.1	1.432	70.0	Severe
Decylisononyl dimethyl ammonium chloride ^b	89.9	1.600	114.0	Severe
Benzalkonium chloride (10%) ^b	120.9	2.640	160.5	Severe
Benzalkonium chloride (5%) ^a	69.4	3.660	124.3	Severe
Triton X-100 (5%) ^a	8.8	1.390	29.6	Moderate
Solids				
Chlorhexidine ^a	122.7	0.024	123.0	Severe
Dibenzoyl-L-tartaric acid ^a	77.9	-0.001	77.8	Severe
Imidazole ^a	69.5	2.273	103.5	Severe
Ammonium nitrate ^a	5.8	0.020	6.6	Mild
1-Napthalene acetic acid ^a	160.3	0.015	160.5	Severe
Sodium oxalate ^a	18.2	0.055	19.0	Mild
2,4-Dichloro-5-sulphamoylbenzoic acid ^a	34.8	0.101	35.3	Moderate
Aluminum hydroxide ^a	23.4	0.011	23.6	Mild
Historical Positive Control Ranges				
Historical positive control range ^d :	30.9 ± 4.6	1.375 ± 0.3	51.6 ± 6.2	Moderate
Ethanol (100%), (mean ± std. dev.)	<i>n</i> = 1171			
Historical positive control range ^d : Imidazole (20% suspension)	72.3 ± 16.5	1.774 ± 0.4	99.2 ± 15.2	Severe
4-h exposure for solids, (mean ± std. dev.)	<i>n</i> = 205			

Note: Liquid chemicals are tested for a 10-min exposure, followed by a 120-min postexposure incubation. Solid chemicals are tested as a 20% aqueous dilution for a 4-h exposure.

^a Data from Schrage et al.,⁸⁷.

^b Data from Sina et al.,⁶⁵.

^c Anionic surfactants induce little increase in opacity relative to the damage caused. Permeability scores are used to predict damage.⁸⁸

^d Historical control range from the Institute for In Vitro Sciences, Inc., Gaithersburg.

TABLE 5.16
Sample Protocol for the Time Course Assay Using the Human Corneal Epithelium
Construct: EpiOcular™
Theory

Ocular irritancy of many classes of chemicals involves the cytotoxicity to the cells of the eye. The prediction of ocular irritancy of a test article is determined by the exposure time of a test article required to reduce cell viability to 50% of control viability. Cell viability is measured by the NADH-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) in the living cells and is expressed as relative viability of the treated to untreated (negative control) cultures.⁸⁹⁻⁹¹

Applications and Use

- Tissue constructs are particularly suited for very mild to moderately toxic materials.
- Test materials are applied topically at formulation strength.
- Both water-soluble and water-insoluble formulations can be tested.
- Creams, pastes, highly viscous materials, and powders otherwise precluded from testing in other models are compatible with this system.
- Cells are of human origin and can be induced to express and release inflammatory mediators.

Experimental Procedure
Receipt and Preparation of Cultures

- Each culture is removed with sterile forceps from the agarose gel in the sterile shipping tray, inspected, and transferred to an AM in a 6-well plate. The EpiOcular cultures will be incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a humidified atmosphere of $5^{\circ}\text{C} \pm 1\%$ CO_2 in air for at least 1 h prior to dosing.

Assay Procedure

- The test chemicals/formulations may be tested neat or at end-use concentrations by topical application. Pastes and highly viscous materials may be “creamed” to effect application.
- The positive control is 0.3% Triton X-100 and is exposed for 5, 15, and 45 min.
- The negative control is sterile, deionized water generally exposed concurrently with the longest and shortest exposure times of the test or positive control articles.
- 100 μL (liquids) or 30 mg (solids) of the test or control article is applied topically onto the tissue surface.
- The cultures are returned to the incubator for the appropriate exposure times. Generally, a minimum of four exposure times, ranging from 1 to 240 min, is selected. For extremely mild materials routinely applied near the eyes, custom exposure times of up to 24 h may be selected.
- After the appropriate exposure time, the test articles are washed from the cultures using DPBS.
- The cultures are transferred to holding wells containing 5 mL of assay medium for a 10–20 min rinse soak.
- The cultures are transferred to wells containing 0.3 mL of MTT reagent (1 mg/mL) and incubated for 3 h.
- After incubation, the cultures are washed with DPBS and extracted in 2 mL of isopropanol for 2 h, with shaking.
- 200 μL of each extraction solution is transferred to a 96-well plate and the absorbance at 550 nm (OD_{550}) recorded.

Data Evaluation

- The relative survival is determined by comparing the mean-corrected OD_{550} of the test article-treated wells to the mean-corrected OD_{550} of the negative control wells.
- Exposure time response curves may be plotted with the percentage of control on the ordinate and the test article exposure time on the abscissa.
- The ET_{50} (the time of exposure to the test article that reduces MTT conversion by 50%) is determined by interpolation from the exposure time response curves.
- Occasionally, a test article may directly reduce the MTT, giving erroneous results. A direct MTT reduction test is performed as a pre-screen, and “killed tissue” controls may be assayed concurrently.
- *Interpretation:* Exposure response curves and ET_{50} values are compared between test formulations as well as benchmarks, where applicable. Several prediction models for interpreting data from a range of chemical classes are under development.⁹⁰

TABLE 5.17

Sample Data from the Time Course Assay Using the Human Corneal Epithelium Construct: EpiOcular™

Test Materials ^a	ET ₅₀ (min) ^b	Draize MMAS ^c
PPG-5 Ceteth-20	180	0.7
Polyoxyethyleneoleyl ether (10%)	240	9.6
Imidazolium compound (5%)	>240	1
Sodium linear alkylbenzenesulfonate	6.9	45
Adult shampoo	9	36
Disinfectant cleaner	5.6	40
Cosmetic Product Category Testing ^d	Mean ET ₅₀ (min)	Range (min)
Adult shampoo (10%), <i>n</i> = 9	27.0	17.1–35.1
Children's shampoo (10%), <i>n</i> = 7	58.2	37.9–135.4
Liquid eyeliner (100%), <i>n</i> = 15	532.2	64–1440
Mascara (100%), <i>n</i> = 20	655.1	199–1080
Eye cream (100%), <i>n</i> = 11	581.8	102–1200
Adult lotion/cream (100%), <i>n</i> = 17	511.4	234–1440
Sunscreen (100%), <i>n</i> = 15	673.6	252–1440
Baby lotion (100%), <i>n</i> = 2	1220.0	1220
Adult hair conditioner (100%), <i>n</i> = 5	279.6	100–530
Historical positive control range: Triton X-100 ^e (0.3%)	27.2 ± 11.5 <i>n</i> = 631	

^a Materials tested neat *in vivo* unless indicated.^b All materials tested at 20% of the indicated concentration that was used *in vivo*. Data from Blazka et al.,⁷⁷.^c Draize modified maximum average scores taken 24 h or more after treatment.^d Data from McCain et al.,⁹².^e Historical control range from the Institute for In Vitro Sciences, Inc., Gaithersburg.

TABLE 5.18

Sample Protocol for the EIT Using the EpiOcular™ Human Corneal Epithelium Construct**Theory**

Ocular irritancy of many classes of chemicals involves the cytotoxicity to the cells of the eye. The EIT evaluates the potential ocular irritancy of a test chemical as a function of the reduction in cell viability after a fixed test chemical exposure to the EpiOcular™ human corneal epithelium construct. Cell viability is measured by the reduction of MTT and is expressed as a percentage relative to untreated (negative control) cultures.⁷⁹

Applications and Use

- For regulatory classification and labeling to identify eye irritants, and to discriminate chemicals not classified as eye irritants from all classes of eye irritant chemicals, according to the UN GHS (No category vs. Category 1/2A/2B).
- Test chemicals are applied topically on the corneal epithelium. Mixtures and formulations may be tested undiluted.
- Proposed for use in a tiered approach in combination with organotypic models (e.g., BCOP) to discriminate between Category 1 and 2 materials.

Experimental Procedure**Receipt and Preparation of Cultures**

- On the day of receipt, the tissues are placed at RT for ~15 min. Each tissue is removed with sterile forceps from the agarose gel, inspected, and transferred to 1.0 mL AM in a 6-well plate. The EpiOcular tissues will be incubated at 37°C ± 1°C in a humidified atmosphere of 5% ± 1% CO₂ in air (standard culture conditions) for 1 h.
- The medium under the tissues is replaced with fresh warmed (37°C)AM, and the tissues are incubated overnight at standard culture conditions (16–24 h).

Assay Procedure

- Prior to dosing, the tissues are prewet with 20 µL of Ca²⁺ Mg²⁺-free DPBS for 30 ± 2 min at standard culture conditions.
- After 30 min, liquid test chemicals and controls are tested by applying 50 µL topically onto the EpiOcular tissues.
- (Exposure conditions for solid test chemicals are still being finalized during the time of this study. Please refer to the finalized validation protocol for details.)

(continued)

TABLE 5.18 (continued)
Sample Protocol for the EIT Using the EpiOcular™ Human Corneal Epithelium Construct

- The negative control is sterile, deionized water.
- The positive control is methyl acetate.
- Treated tissues are incubated for 30 ± 2 min at standard culture conditions.
- After treatment, the test chemicals and controls are rinsed from the tissues by the following method:
- Treated tissues will be removed from the 6-well plates and the test or control chemicals are decanted from the tissue surface onto a clean absorbent material.
- The tissues are dipped into the first of the three beakers of clean DPBS, swirled for at least 2 s, decanted, and then the rinsing repeated in the remaining two beakers of DPBS.
- The cultures are transferred to holding wells containing 5 mL of assay medium for a 12 ± 2 min rinse soak.
 - After the postrinsing soak, medium is decanted from the tissue, the insert is blotted and transferred to a well of a 6-well plate containing 1 mL of warm AM. The tissues are incubated for a 120 ± 15 min posttreatment expression period at standard culture conditions.
- After the incubation, the tissues are gently blotted and transferred to wells containing 0.3 mL of MTT reagent (1 mg/mL) and incubated for 3 h at standard culture conditions.
- After the MTT incubation, the tissues are extracted in 2 mL of isopropanol for 2 h with shaking.
- The tissues are pierced and 2 × 200 µL samples of extraction solution from each tissue are transferred to a 96-well plate and the absorbance at 570 nm (OD₅₇₀) recorded.

Data Evaluation

- Relative viability is determined by comparing the mean blank-corrected OD₅₇₀ of the test chemical-treated tissues to the mean blank-corrected OD₅₇₀ of the negative controls.
- A prediction model has been developed by MatTek Corporation to determine the ocular irritation classification as follows:
- If the mean tissue viability is >50% relative to negative control, the test chemical is predicted to be *NI (GHS not classified)*.
- If the mean tissue viability is ≤50% relative to negative control, the test chemical is predicted to be *irritant*.
- Occasionally, a test article may directly reduce the MTT, giving erroneous results. Similarly, highly colored chemicals with absorbance at 570 nm may be persistent on the tissues, affecting the final OD₅₇₀ determination. A direct MTT reduction test is performed as a prescreen, and “freeze-killed tissue” controls may be assayed concurrently. The results of the freeze-killed tissue controls may be used to correct the OD₅₇₀ values of the test chemical-treated tissues.

TABLE 5.19
Sample Data from the EIT Using the Human Corneal Epithelium Construct: EpiOcular™

Eye Irritation Test Assay (50 µL [Liquids] with a 30-min Exposure and 120-min Postexposure Incubation)	Mean Tissue Viability ≤50% = Irritant (GHS 1 or 2) Mean Tissue Viability >50% = NI Mean ± SD	GHS Category (<i>In Vivo</i>)
Benzalkonium chloride (10%)	7.2 ± 0.29	Category 1
Butanol	4.52 ± 0.77	Category 2A/2B
Acetone	15.85 ± 3.85	Category 2A/2B
Ethanol	12.68 ± 3.64	Category 2A/2B
Methyl acetate (positive control)	34.97 ± 1.07	Category 2A/2B
Tween 20	102.76 ± 2.97	Not classified
Glycerol	102.22 ± 0.38	Not classified
Polyethylene glycol 400	95.19 ± 0.99	Not classified
Ethanol (10%)	96.89 ± 0.46	Not classified
Dodecane	97.60 ± 1.19	Not classified
Triton X-100 (1%)	17.98 ± 4.84 (false-positive)	Not classified

Source: Data taken from Kaluzhny, Y. et al., *Alt. Animal Exp.*, 39, 339, 2011.

**ASSESSMENT OF DERMAL
IRRITATION POTENTIAL**

Selection of the most appropriate dermal model depends on the question being asked and the cells/tissue available. Many models are used for both irritation and efficacy assessment. Often, the first step in assessing active ingredients may be

to use a simple cell-based assay to identify hazard or effect, without regard to actual penetration and exposure in the skin. Most major cell types from human skin are available for cell-based studies. More complex exposure kinetics require *ex vivo* skin or 3D-engineered human skin construct model systems that provide both the barrier properties of skin and the necessary target cells, and offer a measure of the actual

response expected in skin. Specialized assays using cell and tissue construct models have been developed for phototoxicity and corrosivity/skin irritation, and the latter has received regulatory approval (OECD).

For irritation assessment, time-to-toxicity protocols using 3D reconstructed human epidermis models (RhE) were initially developed, which spanned the spectrum of skin irritation effects, from predicting potentially corrosive materials to discriminating among extremely mild formulations. The concept is simply based upon the expectation that corrosive or irritant materials are tolerated by the test system for relatively short exposure times, while progressively milder materials are tolerated for commensurately longer exposure times. This tolerance is a function of both the inherent cytotoxicity of the material and its ability to penetrate the stratum corneum. Regulatory protocols were subsequently developed from this concept for classification and labeling purposes by focusing upon the exposure time range relevant to the irritation classification. Corrosivity protocols were established using fixed exposure times, generally at quite short 3, 60, and 240 min exposures, similar to the exposure/evaluation times used *in vivo*. The prediction models essentially returned corrosive or noncorrosive predictions (and in some protocols, resolve among corrosive subcategories) depending upon whether tissue viabilities at each of the exposure times were found to be greater or less than established threshold response levels. Subsequently, similar approaches were used to develop skin irritation tests for predicting Globally Harmonized System (GHS) 2 skin irritants. In the skin irritation tests, tissues were exposed at a fixed exposure somewhat longer than that found to be predictive of corrosive materials, and again the prediction models essentially returned irritant or NI predictions (by GHS classification and labeling categories) depending upon whether tissue viabilities for the fixed exposures were found to be greater or less than 50%.

Each of these validated regulatory methods is designed to specifically test for a single category or classification of skin corrosion or irritation, rather than provide a continuum of potential responses over the skin corrosion/irritation spectrum. But by combining assays in a tiered approach, one can readily assess whether a chemical is a skin irritant or corrosive.

Three-dimensional tissue constructs offer significant advantages over isolated cell-culture systems for determining dermal toxicity. They have a greater degree of cell-cell interaction than cell culture models, exhibit progressive differentiation similar to the native counterpart, and have an intact and functional *stratum corneum*. Although all RhE models have a functional *stratum corneum*, the barrier properties among these models vary among tissue offerings, and they do not yet exhibit the degree of barrier function found in native healthy skin.^{93–96}

These RhE *in vitro* assays can be used in a tiered testing strategy for regulatory purposes to predict the skin corrosion

or irritation potential of test materials, following a top-down or bottom-up strategy (similar to that discussed earlier for the prediction of eye irritation potential). The top-down strategy may be employed when the test material is expected to be corrosive or highly irritant, by using one of the corrosivity test methods. If the test material is found to be corrosive in one of these assays, then it may be labeled as corrosive. Furthermore, if the assay is designed to indicate specific corrosive packing groups (i.e., discrimination between GHS category 1A, or GHS category 1B/C), the test material may also be so classified. Since these specific corrosion protocols do not provide sufficient additional information on how mild a *noncorrosive* test material may be, a noncorrosive test material must be further tested in one of the validated *in vitro* skin irritation tests for final classification.

The bottom-up strategy can be used if the test material is not expected to be corrosive, using one of the skin irritation test methods validated for this purpose. If the test material is not found to be a skin irritant, then the test material will not require any further skin irritation testing for submission to regulatory agencies utilizing the GHS system. However, if the test material gives a positive result with the skin irritation test, then the test material must also be tested with one of the aforementioned corrosion tests prior to labeling the materials. Materials that are predicted to be GHS category 2 positive, but not corrosive, are labeled as GHS category 2 skin irritants, while those that are predicted to be both GHS category 2 positive and corrosive (i.e., GHS category 1 positive) are labeled as corrosive. This labeling scheme is used because the skin irritation test protocols will predict whether a material is a skin irritant or not, but if the test material is found to be an irritant, these skin irritation test protocols do not provide additional information as of how irritant or potentially corrosive a material may be.

To address the irritancy ranking and to meet the typical needs of product development groups charged with developing increasingly milder products, other nonregulatory protocols using these RhE models have been designed so that the researcher can use them to differentiate among similar product candidates with a high level of precision heretofore not possible with animal models. These protocols generally use the time course (time-to-toxicity) approach, and are based on the exposure time it takes to reduce tissue viability by 50% (ET₅₀) as measured by the tissue's ability to reduce MTT.⁷ The exposure times are selected to cover the wide continuum of skin irritation events from very short exposure times (predictive of corrosive chemicals) to long exposure times of up to 24 h (predictive of very mild, nonirritating formulations). These methods can readily be enhanced to increase resolution to measure modest improvements in candidate formulations by modifying the test system exposure kinetics (exposure times, doses, exposure concentrations). Using this strategy, the *in vitro* skin models can provide vastly more information on relative and comparative skin irritation or skin tolerance than the regulatory tests do, and can provide

the fine resolution between similar formulations to provide rank order or irritant potential typically required to support product development and refinement programs in the personal care and household products industries.

Overall, the RhE models serve many testing purposes such as hazard identification and labeling of chemicals, transport of dangerous substances (industrial chemicals), labeling of finished products (cleaning agents, household products), occupational safety/industrial hygiene, and safety testing and risk assessment of raw ingredients or final formulations of personal care and cosmetic products.

The phototoxic potential of a test chemical can be evaluated using the cell-based 3T3 neutral red uptake phototoxicity assay.⁹⁷ The assay, used for hazard assessment, compares the dose-dependent toxicity of a test chemical in the presence and absence of UVA exposure in cultures of normal Balb/c 3T3 mouse fibroblasts. Indeed, these cells are neither human nor skin-derived, but are used in the test primarily because

they are readily available, are relatively easy to culture, and have historically been used in cytotoxicity tests. The assay can identify phototoxic potential of chemicals and ingredients and provides a mechanistic model of UVA-induced reactive chemical toxicity. However, there are limitations to the test system. For example, since the assay is a monoculture system, it does not model exposure kinetics such as skin penetration, nor does it address metabolism or distribution of the chemical of interest. Furthermore, there are classic limitations of testing hydrophobic materials in the aqueous dilution-based assay, and the interactions of multiple ingredients in complex formulations can be challenging. Fortunately, RhE model can be used for evaluating the phototoxic potential of lipophilic chemical ingredients or complex formulations, particularly for products targeted for skin exposure.⁹⁸ Indeed, these latter models can be very useful for evaluating the relevancy of irritant and photo-absorbing ingredients at final formulation concentrations (Tables 5.20 through 5.30).

TABLE 5.20

Cell/Tissue-Based Models for the Evaluation of Chemical Action on the Skin

Screening of individual chemicals/ingredients for toxicity or efficacy in monolayer culture systems:

- Models serve to assess the *potential* for the material to act on the target cells without regard to the actual exposure that might be achieved *in vivo*.
- The measurement is independent of penetration into skin since the cells are exposed directly in aqueous medium.
 - Model systems composed of monolayer cell cultures of individual cell types (e.g., keratinocytes, dermal fibroblasts, melanocytes, dendritic cells)
 - Test material prepared in aqueous medium and usually over a series of dilutions
 - Requires that ingredient be water-miscible
 - Aqueous insoluble ingredients may be tested in a nontoxic solvent on a tissue construct
- Assay end points may include the following:
 - Direct toxicity (immediate and delayed)
 - Induction/inhibition of inflammatory mediator expression
 - Phototoxicity
 - Inhibition of differentiated function (e.g., collagen synthesis/release)

Assessment of ingredients, mixtures, and formulations where the goal is to predict the action on skin *in vivo*:

- Action of the formulation/active ingredients is mediated by their ability to penetrate through the stratum corneum.
 - Tissue constructs, with a functional stratum corneum, are the models of choice
 - Epidermal-only or epidermal/dermal “full-thickness” constructs
 - Allow direct application of the test material to the “dry” stratum corneum
 - Time to observed action depends on the penetration as well as the “innate” toxicity/potential activity of the material/formulation
 - Applications
 - Direct toxicity (immediate and delayed)
 - Induction/inhibition of inflammatory mediator expression
 - Phototoxicity
 - Inhibition of differentiated function
 - Additional end points from specialized systems with multiple cell types:
 - Inhibition of melanin deposition in keratinocyte/melanocyte constructs
 - Epidermal–dermal cell interaction (e.g., balanced cytokine expression or induction of proteases)
-

TABLE 5.21
Examples of Monolayer Cell-Based Assay Systems for Assessing Chemical Action on the Skin

Cell Type	Culture System ^a	Applications	End Point(s)
Balb/c 3T3 fibroblasts (mouse)	Serum-containing medium	Phototoxicity ⁹⁹	Neutral red uptake
Human keratinocytes	Serum-free medium derived from MCDB 153 ³⁰	Direct cytotoxicity ¹⁰⁰	Neutral red uptake
	Serum-free medium derived from MCDB 153 without hydrocortisone	Phototoxicity Stimulation of expression/release of inflammatory mediators ¹⁰¹ Anti-inflammatory action decreased expression/release of inflammatory mediators	Neutral red uptake ELISA: TNF α , IL-1 α , IL-8 ELISA: TNF α , IL-1 α , IL-8
Human dermal fibroblasts	Serum-containing medium Ascorbic acid required	Direct cytotoxicity Suppression/stimulation of collagen synthesis ¹⁰²	Tetrazolium dye reduction ELISA for procollagen c-peptide
Human melanocytes	Various media including low serum formulations	Melanin synthesis ¹⁰³	Changes in tyrosinase activity
Human microvascular endothelial cells	Low serum medium	Proinflammatory responses ¹⁰⁴	Upregulation of adhesion proteins
Human dendritic cells (from bone marrow stem cells)	Proprietary formulations that induce differentiation	Interaction with antigenic/sensitizing agents ¹⁰⁵	Cytokine expression (e.g., IL-1 β)

^a See cell supplier literature for the recommended culture medium.

TABLE 5.22
Neutral Red Uptake Phototoxicity Assay in BALB/c 3T3 Mouse Fibroblasts

Theory

Cells are exposed to serial doses of the test article in the presence or absence of a nontoxic flux of UVA light. A phototoxin transfers light energy to the cells in a deleterious manner, leading to increased cytotoxicity relative to the doses of the test article alone. The uptake of neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) by Balb/c 3T3 mouse fibroblast cell cultures is used to measure changes in cell viability relative to control values.^{106–108}

Applications and Use

- The assay provides a mechanistically-relevant measure of a chemical's phototoxic potential. This makes the assay ideal for screening for hazard assessment of individual ingredients and chemicals.
- The assay requires test chemicals to be diluted in an aqueous vehicle. Whereas aqueous-soluble chemicals can be readily tested in the system, hydrophobic/lipophilic chemicals and complex formulations may not be adequately tested.

Experimental Procedure

Target Cell Preparation

- Stock Balb/c 3T3 cell cultures are maintained at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a humidified atmosphere containing $5\% \pm 1\% \text{CO}_2$.
- Cells are subcultured when the stock culture is 50%–80% confluent.
- A cell suspension is prepared to yield 1.0×10^5 cells/mL.
- 100 μL ($\sim 10,000$ cells per well) of the cell suspension is added into the designated wells of the 96-well bioassay plate.
- The cultures are incubated for ~ 24 h.
- Since UV sensitivity of the cells increases with aging, cells are used at passage numbers <100 . Stock cells are cultured in the absence of antibiotics and new stock cells are initiated from the frozen working bank every 3 months.

Sample and Positive Control Preparation (prepared on the day of treatment)

- On the day of dosing, the test chemical is suspended in EBSS or HBSS ($2 \times$ concentration). Intermediate solvents (DMSO, ethanol, or acetone) may be used to prepare initial stock dilutions with subsequent dilution into the aqueous medium (final maximum solvent concentration of 0.5%–1%).
- A total of eight dilutions of the test chemical are made for the dose range-finding assay (ranging from 1000 to 0.3 $\mu\text{g/mL}$ in 1/2 log increments), and a total of eight dilutions are made for the definitive assays based on the results of the dose range-finding assay.
- Chlorpromazine is used as the positive control. Eight concentrations (ranging from 100 to 1.63 $\mu\text{g/mL}$ for the plate not exposed to UVA light (dark) and from 9.53 to 0.156 $\mu\text{g/mL}$ for the plate exposed to UVA light) are tested.

Assay Procedure

- At 24 h after seeding the cells into 96-well plates, the growth medium is removed, cells rinsed once with 125 μL EBSS or HBSS, and the appropriate wells are refed with 50 μL of the dosing vehicle (EBSS/HBSS). The outer wells are refilled with 100 μL of isotonic solution to maintain the humidity.
- Two plates are designated for each test material: one plate for determination of cytotoxicity (dark) and the second plate for determination of phototoxicity (UVA); 50 μL of the test and control article dilutions are added to the appropriate wells yielding the final treatment dose. The cells are exposed to the test article dilutions for approximately 1 h.
- After the 1-h treatment, one plate is exposed to $1.7 \pm 0.1 \text{ mW/cm}^2$ of UVA light and the second plate is placed in the dark at room temperature, both incubated for 50 ± 2 min. The plates are then rinsed at least once and refed with 100 μL of assay medium (growth medium supplemented with penicillin/streptomycin).
- Prior to the neutral red addition, the wells are evaluated microscopically for cytotoxicity.
 - Approximately 24 h after the posttreatment incubation, the AM is removed from the wells and replaced with 100 μL of neutral red solution (50 $\mu\text{g/mL}$ neutral red in AM). The 100 μL of isotonic solution is added to the blank wells (outer wells).
- The 96-well plates are returned to the incubator for 3 h to allow the neutral red uptake within the viable cells.
- After 3 h, the neutral red solution is removed and rinsed once with 250 μL EBSS or HBSS. Next, 100 μL of solvent (ethanol and acetic acid) is added.
- The neutral red is extracted from the cultures for at least 20 min at room temperature while shaking.
- The absorbance of the neutral red at 550 nm (OD_{550}) is measured with a 96-well plate reader.

Data Evaluation

- The relative survival of each treatment group is determined by comparing the mean-corrected OD_{550} of the test article-treated wells to the mean-corrected OD_{550} of the solvent control wells.
- Dose–response curves may be plotted with the percent of control on the ordinate and the test article doses on the abscissa.
- The IC_{50} (the concentration of the test article that inhibits the uptake of neutral red by 50%) is determined by interpolation from the dose–response curves.

TABLE 5.22 (continued)

Neutral Red Uptake Phototoxicity Assay in BALB/c 3T3 Mouse Fibroblasts

- A photo-irritancy factor (PIF) value and a mean photo-effect (MPE) value were calculated for each test article using the software program PHOTOTOX (version 2.0) software provided by ZEBET (Berlin, Germany). The PIF is determined by comparing the IC_{50} in the absence of UVA exposure (–UVA) with the IC_{50} in the presence of UVA exposure (+UVA) to determine the “factor” difference.

$$PIF = IC_{50}(-UVA)/IC_{50}(+UVA).$$

A PIF of >5 is predictive of phototoxic potential; a PIF of <5 but ≥ 2 is predictive of “probable” phototoxic potential; and a PIF of <2 is predictive of no phototoxic potential.

- The MPE measures the effect of UV exposure over a range of concentrations, and essentially compares the difference in the areas under the response curves for the test chemical treated in the presence of UVA exposure to that of the chemical treated in the absence of UVA exposure. The theoretical dynamic range of the MPE varies from 0 to 1.0.

A MPE of ≥ 0.15 is predictive of phototoxic potential; a MPE of <0.15 but ≥ 0.1 is predictive of “probable” phototoxic potential; and a MPE of <0.1 is predictive of no phototoxic potential.⁹⁷

TABLE 5.23

Sample Data from the Neutral Red Uptake Phototoxicity Assay in BALB/C 3T3 Mouse Fibroblasts

Test Articles ¹⁰⁹ (Tetracycline Derivatives)	IC_{50} (μg/mL) (with UVA)	IC_{50} (μg/mL) (without UVA)	Photo-Irritancy Factor ^a	Mean Photo-Effect ^b
Derivative A	>100	>100	None	0.032
Minocycline	58.2	>100	>1.7	0.121
Tetracycline	17.8	>100	>5.62	0.703
Doxycycline	3.24	74.9	23.0	0.794
Derivative D	0.33	>100	>305	0.691
Historical positive control range: Chlorpromazine in HBSS ^c ($n = 287$)	1.06 ± 0.74	21.9 ± 13.4	21.6	0.548 ± 0.168

^a $PIF = IC_{50}(-UVA)/IC_{50}(+UVA)$. A PIF of >5 is predictive of phototoxic potential; a PIF of <5 but ≥ 2 is predictive of “probable” phototoxic potential; and a PIF of <2 is predictive of no phototoxic potential.

^b MPE, mean photo-effect. A MPE of >0.15 is predictive of phototoxic potential; a MPE of <0.15 but ≥ 0.1 is predictive of “probable” phototoxic potential; and a MPE of <0.1 is predictive of no phototoxic potential.

^c Historical positive control range from the Institute for In Vitro Sciences, Inc., Gaithersburg.

Reference Material Data From OECD Test Guideline 432⁹⁷, Table 1

Chemicals	Photo-Irritancy Factor	Mean Photo-Effect	Absorption Peak (nm)	Solvent
Amiodarone HCl	>3.25	0.27–0.54	242–300	Ethanol
Chlorpromazine HCl	>14.4	0.33–0.63	309	Ethanol
Norfloxacin	>71.6	0.34–0.90	316	Acetonitrile
Anthracene	>18.5	0.19–0.81	356	Acetonitrile
Protoporphyrin 1X, Disodium	>45.3	0.54–0.74	402	Ethanol
L-Histidine	No PIF	0.05–0.10	211	Water
Hexachlorophene	≤ 1.7	0.00–0.05	299–317	Ethanol
Sodium lauryl sulfate	≤ 1.9	0.00–0.05	No absorption	Water

TABLE 5.24
Examples of Tissue Models for Skin Studies

General Description	Applications	Examples of End Points Measured
Excised Skin		
Human skin: Partial thickness, living or previously frozen ¹¹⁰	Percutaneous absorption studies; live tissue allows assessment of biotransformation	Quantitative measurements of radiolabeled or nonlabeled materials in the receiver fluid
Human skin: Full thickness, living	Cytotoxicity/irritancy/corrosion	Transepithelial resistance Transepithelial water loss Cell viability measured by vital dye reduction
	Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products
	Biotransformation	Quantitative measurements of radiolabeled or nonlabeled materials in the receiver fluid
Porcine skin: Partial thickness, living or previously frozen	Percutaneous absorption studies	Quantitative measurements of radiolabeled or nonlabeled materials in the receiver fluid
Rodent skin: Living or previously frozen ⁶⁰	Cytotoxicity/irritancy/corrosion	Transepithelial resistance Transepithelial water loss
Tissue Constructs⁶¹		
CellSystems EST-1000	Cytotoxicity/irritancy/corrosion	Cell viability measured by vital dye reduction
Epidermal tissue only with a developed stratum corneum ¹¹¹	Phototoxicity Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products
MatTek Corporation: EpiDerm™	Cytotoxicity/irritancy/corrosion	Cell viability measured by vital dye reduction
Epidermal tissue only with a developed stratum corneum ¹¹²	Phototoxicity Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products
SkinEthic: EPISKIN™	Cytotoxicity/irritancy/corrosion	Cell viability measured by vital dye reduction
Epidermal tissue only with a developed stratum corneum ¹¹³	Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products
SkinEthic™ RHE	Cytotoxicity/irritancy/corrosion	Cell viability measured by vital dye reduction
Epidermal tissue only with a developed stratum corneum ¹¹⁴	Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products
StrataTech Corp. StrataTest® Full thickness skin tissue comprised of an epidermis with functional stratum corneum and dermal fibroblast dermis ¹¹⁵	Cytotoxicity/irritancy/corrosion Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	Cell viability measured by vital dye reduction ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products
Japan Tissue Engineering Co., Ltd., LabCyte EPI-MODEL ¹¹⁶	Cytotoxicity/irritancy/corrosion	Cell viability measured by vital dye reduction
Epidermal tissue only with a developed stratum corneum	Regulation of inflammatory mediator (e.g., arachidonic acid products and cytokines)	ELISA or HPLC assays for arachidonic acid products RT-PCR for mRNA ELISA for protein products

TABLE 5.25
Assays for Skin Corrosion

Models	Basis of the Assay	End Point Measured	Scoring	Notes
Nontissue Methods				
Corrositex ^{TM27}	Penetration of the test material through a collagen biomatrix “biobarrier”	Color change in the chemical detection solution below the biobarrier	Time required to penetrate biobarrier	Generally applicable only to acids, acid derivatives, and bases; absence of a test article-induced color change in the chemical detection system precludes use of the assay
Tissue-Based Methods				
MatTek Corp. EpiDerm	Damage to cells in a 3D artificial human skin construct after topical exposure to test materials	Number of viable cells as estimated by uptake and reduction of the dye MTT	Specific reductions in tissue viability after fixed exposure times predictive of corrosive or irritant effects	Applicable to most chemicals without regard to their physical state (solids or liquids) or water solubility
SkinEthic RHE	Damage to cells in a 3D artificial human skin construct after topical exposure to test materials	Number of viable cells as estimated by uptake and reduction of the dye MTT	Specific reductions in tissue viability after fixed exposure times predictive of corrosive or irritant effects	Applicable to most chemicals without regard to their physical state (solids or liquids) or water solubility
CellSystems EST-1000	Damage to cells in a 3D artificial human skin construct after topical exposure to test materials	Number of viable cells as estimated by uptake and reduction of the dye MTT	Specific reductions in tissue viability after fixed exposure times predictive of corrosive or irritant effects	Applicable to most chemicals without regard to their physical state (solids or liquids) or water solubility
SkinEthicEPISKIN ⁶⁹	Damage to cells in a 3D artificial human skin construct after topical exposure to test materials	Number of viable cells as estimated by uptake and reduction of the dye MTT	Specific reductions in tissue viability after fixed exposure times predictive of corrosive or irritant effects	Applicable to most chemicals without regard to their physical state (solids or liquids) or water solubility
J-TEC LabCytEPI-MODEL	Damage to cells in a 3D artificial human skin construct after topical exposure to test materials	Number of viable cells as estimated by uptake and reduction of the dye MTT	Specific reductions in tissue viability after fixed exposure times predictive of corrosive or irritant effects	Applicable to most chemicals without regard to their physical state (solids or liquids) or water solubility
Rat skin transcutaneous electrical resistance	Loss of normal stratum corneum integrity and barrier function after topical exposure to test material	Reduction in transcutaneous electrical resistance	Treatment time required to reduce transcutaneous electrical resistance below a predetermined threshold level	Applicable to most chemicals without regard to their physical state (solids or liquids) or water solubility

TABLE 5.26**Sample Protocol for the Corrosivity Assay Using the EpiDerm™ Skin Model****Theory**

Skin corrosivity is the chemical-mediated irreversible skin damage typically characterized by necrosis and scar formation into the dermis. The *in vitro* skin corrosion assay is used to predict the corrosive potential of a test chemical by determining the decrease in cell viability in the reconstructed human epidermis model after fixed exposures. Relative cell viability is determined by comparing the cellular reduction of MTT in treated and control cultures.

Applications and Use

- Approved methods described in OECD Test Guideline 431²⁶
- The EpiDerm model is composed of human keratinocytes stratified into a 3D epidermal structure consisting of several layers, including a functioning *stratum corneum*. Similar models may also be employed (see Table 20.24).
- Used to predict skin irritation of neat substances according to the Globally Harmonized System GHS 1 or noncorrosive.
- EpiDerm™ is suited to address the corrosive potential of test materials.
- Test chemicals are applied topically at formulation strength.
- Suited for both water-soluble and insoluble formulations.

Experimental Procedure**Receipt and Preparation of Cultures**

- Each culture is removed from the agarose gel with sterile forceps, inspected, and transferred to a prelabeled 6-well plate containing 0.9 mL of AM per well. The EpiDerm™ cultures will be incubated at 37°C ± 1°C in a humidified atmosphere of 5% ± 1% CO₂ in air (standard culture conditions) for at least 1 h prior to dosing.

Assay Procedure

- Test materials are tested neat by topical application onto the *stratum corneum*.
- The positive control is 8N KOH
- The negative control is sterile, deionized water.
- 50 µL (liquids) or 25 mg (solids) of the test or control materials are applied topically onto the tissue surface.
- The cultures are treated for two fixed exposure times of 3 and 60 min. The 60-min exposures are incubated at standard culture conditions.
- After the appropriate exposure time, the test articles are rinsed from the cultures using Ca²⁺ and Mg²⁺-free DPBS.
- The cultures are transferred to wells containing 0.3 mL of MTT reagent (1 mg/mL) and incubated for 3 h.
- After incubation, the cultures are blotted on absorbent paper and extracted in 2 mL of isopropanol for 2 h, with shaking.
- 200 µL of each extraction solution is transferred to a 96-well plate and the absorbance at 550 nm (OD₅₅₀) recorded.

Data Evaluation¹¹⁷

- The relative survival is determined by comparing the mean-corrected OD₅₅₀ of the test chemical-treated tissues to the mean-corrected OD₅₅₀ of the negative control-treated tissues.
- *Prediction model:* Test materials that reduce tissue viability to <50% after a 3-min exposure and/or <15% after a 60-min exposure are classified corrosive. Test material responses that result in tissue viabilities of ≥50% after a 3-min exposure and ≥15% after a 60-min exposure are classified noncorrosive.
- Occasionally, a test article may directly reduce the MTT giving erroneous results. A direct MTT reduction test is performed as a prescreen, and “killed tissue” controls may be assayed concurrently.

Note: See the OECD Test Guideline for exposure conditions for the other *in vitro* skin models.

TABLE 5.27**Sample Protocol for the Skin Irritation Test Using the EpiDerm™ Skin Model****Theory**

For many classes of materials, irritancy is manifested in cell cytotoxicity and/or upregulation of inflammatory mediators. This assay evaluates the potential dermal irritancy of a test chemical as a function of the reduction in cell viability after a fixed test chemical exposure to the EpiDerm™ construct. Cell viability is measured by the reduction of MTT and is expressed as a percentage relative to untreated (negative control) cultures.^{28,112}

Applications and Use

- Approved methods described in OECD Test Guideline 439.
- Used to predict skin irritation of neat substances according to the GHS 2 or no-label.
- The EpiDerm™ model is composed of human keratinocytes stratified into a 3D dermal structure consisting of basal, spinous, and granular layers, including a functioning *stratum corneum* with characteristic lipid lamellae.
- Test chemicals are applied topically at formulation strength.
- Suitable for both water-soluble and insoluble formulations.
- Cells are of human origin and can be induced to express and generate inflammatory response cytokines.

Experimental Procedure**Receipt and Preparation of Cultures**

- Each culture is removed with sterile forceps from the agarose gel, inspected, and transferred to a prelabeled six-well plate containing 0.9 mL of assay medium (AM) per well. The EpiDerm cultures will be incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a humidified atmosphere of $5\% \pm 1\%$ CO_2 in air for 1 h, refed, and incubated for approximately 18 h prior to dosing.

Assay Procedure

- The test materials are tested neat by topical application.
- The positive control is 5% SDS.
- The negative control is sterile, Dulbecco's phosphate-buffered saline (DPBS).
- 30 μL (liquids) or 25 mg (solids) of the test or control article is applied topically onto the tissue surface.
- The cultures are treated for 60 min and incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ during the exposure.
- After exposure, the test articles are rinsed from the cultures using sterile Ca^{++} and Mg^{++} free DPBS.
- The cultures are returned to the incubator for 42 ± 2 h, to allow for the expression of toxic effects.
- The cultures are then transferred to wells containing 0.3 mL of MTT reagent (1 mg/mL) and incubated for 3 h.
- After incubation, the cultures are blotted on absorbent paper and extracted in 2 mL of isopropanol for 2 h, with shaking.
- 200 μL of each extraction solution is transferred to a 96-well plate and the absorbance at 570 nm (OD_{570}) recorded.
- Optional: Medium samples may be collected and prepared to determine IL-1 alpha release.

Data Evaluation

- The relative survival is determined by comparing the mean-corrected OD_{550} of the test article-treated wells to the mean-corrected OD_{550} of the negative control-treated wells.
- The following prediction model is used to evaluate the test article responses:
- Occasionally, a test article may directly reduce the MTT giving erroneous results. A direct MTT reduction test is performed as a prescreen, and "killed tissue" controls may be assayed concurrently.

<i>In Vitro</i> Result	<i>In Vivo</i> Prediction
Mean tissue viability $\leq 50\%$	Irritant (I); GHS 1/2
Mean tissue viability $> 50\%$	NI (not classified)

Note: See OECD Test Guideline 439 for exposure conditions for the other *in vitro* skin models.

TABLE 5.28**Sample Protocol for the Time Course Assay with EpiDerm™ Cultures: MTT End Point****Theory**

For many classes of materials, irritancy/corrosivity is manifested in cell cytotoxicity and/or upregulation of inflammatory mediators. This assay evaluates the potential dermal irritancy of a test article as a function of the exposure time of a test article required to reduce cell viability to 50% of control viability to the EpiDerm™ construct. Cell viability is measured by the reduction of MTT and is expressed as a percentage relative to untreated (negative control) cultures. Cytokine expression may be combined with the cytotoxicity end point to refine the assay further.^{118,119}

Applications and Use

- The EpiDerm model is composed of human keratinocytes stratified into a 3D epidermal structure consisting of several layers, including a functioning stratum corneum. Similar models may also be employed (see Table 20.24).
- The EpiDerm construct is suited to address the sensitivity range from very mild to severely aggressive, or corrosive, materials.
- Test materials are applied topically at formulation strength.
- Suited for both water-soluble and water-insoluble formulations.
- Suitable for testing creams, pastes, highly viscous materials, and powders.
- Cells are of human origin and can be induced to express and generate inflammatory response cytokines.

Experimental Procedure**Receipt and Preparation of Cultures**

- Each culture is removed with sterile forceps from the agarose gel in the shipping container, inspected, and transferred to a prelabeled 6-well plate containing 0.9 mL of AM per well. The EpiDerm tissues are incubated at 37°C ± 1°C in a humidified atmosphere of 5% ± 1% CO₂ in air for at least 1 h prior to dosing.

Assay Procedure

- The test materials are tested neat or at end-use concentrations by topical application to the stratum corneum. Pastes and highly viscous materials may be “creamed” to effect application.
- The positive control is 1.0% Triton X-100 and is exposed for 4 and 8 h.
- Positive controls for modeling inflammatory responses (e.g., croton oil, PMA) may be included.
- The negative control is sterile, deionized water generally exposed concurrently with the longest and shortest exposure times of the test or positive control articles.
- 100 µL (liquids) or 30 mg (solids) of the test or control article is applied topically onto the tissue surface.
- The cultures are returned to the incubator for the appropriate exposure times. Generally, a minimum of four exposure times, ranging from 30 min to 24 h, are selected.
- After the appropriate exposure time, the test articles are rinsed from the cultures using DPBS without Ca²⁺ and Mg²⁺.
- The cultures are transferred to wells containing 0.3 mL of MTT reagent (1 mg/mL) and incubated for 3 h.
- After incubation, the cultures are blotted on absorbent paper and extracted in 2 mL of isopropanol for 2 h, while shaking.
- 200 µL of each extraction solution is transferred to a 96-well plate and the absorbance at 550 nm (OD₅₅₀) recorded.
- Medium samples may be collected and prepared for a variety of cytokine analyses to assess inflammatory responses.

Data Evaluation

- The relative survival is determined by comparing the mean-corrected OD₅₅₀ of the test article-treated wells to the mean-corrected OD₅₅₀ of the negative control-treated wells.
- Exposure time–response curves may be plotted with the percent of control on the ordinate and the test article exposure times on the abscissa.
- The ET₅₀ (the time of exposure to the test article that reduces MTT conversion by 50%) is determined by interpolation from the exposure time–response curves.
- Occasionally, a test article may directly reduce the MTT giving erroneous results. A direct MTT reduction test is performed as a prescreen, and “killed tissue” controls may be assayed concurrently.
- *Interpretation:* Exposure response curves and ET₅₀ values are compared between test formulations as well as benchmarks, if applicable.

TABLE 5.29

Sample Data from the Corrosivity, *In Vitro* Skin Irritation, and Time Course Assays Using the Human Skin Construct EpiDerm™

Test Material Corrosivity Assay (50 μ L Treatment with 3-min Exposure) ^a	Assay End Point Measure Percent Viability Compared with Controls Using MTT End Point (<50% is Corrosive) after a 3-min Exposure	<i>In Vivo</i> Classification
Nitric acid	7.1	Corrosive
Potassium hydroxide (10%)	14.8	Corrosive
Acetic acid (10%)	67.0	Noncorrosive
Oxalic acid	92.6	Noncorrosive
Positive control: KOH (10%)	14.8 \pm 6.6	
Skin Irritation Test Assay (30 μ L [Liquids] 25 mg [Solids] with a 60-min Exposure and 42-h Postexposure Incubation) ^b	Mean Tissue Viability \leq 50% = Irritant (GHS 1 or 2) Mean Tissue Viability >50% = NI Mean \pm SD (three trials of three tissues each)	GHS Category
Isopropanol	90.12 \pm 11.63	Not classified
Methyl stearate	98.74 \pm 4.51	Not classified
Benzylalcohol	5.46 \pm 1.12 (false positive)	Not classified
1-Bromohexane	20.65 \pm 7.92	Category 2
Potassium hydroxide (5% aq)	4.31 \pm 1.02	Category 2
Heptanal	6.36 \pm 1.70	Category 2
Time Course Assay (100 μ L Treatment in Time Course Studies) ^c	ET ₅₀ (min)	<i>In Vivo</i> Classification
1,1,1-Trichloroethane	27 min	Irritant
Sodium lauryl sulfate (20%)	50 min	Irritant
1,6-Dibromohexane	520 min	Mild irritant
3,3'-Dithiodipropionic acid	>1440 min	Nonirritant
Historical positive control range: Triton X-100 (1%) ^d	343 \pm 86 min N = 326	

^a Data from Liebsch et al.,¹¹⁷.

^b Data from Kandarova et al.,¹¹².

^c Data from Fentam et al.,¹¹⁷.

^d Historical control range from the Institute for In Vitro Sciences, Inc., Gaithersburg.

TABLE 5.30**Sample Protocol for the Phototoxicity Assay Using the EpiDerm™ Skin Model****Phototoxicity Assay Using the EpiDerm™ Skin Model****Theory**

This assay is used to evaluate the potential for phototoxic action of the test articles on human keratinocytes in the EpiDerm™ Skin Model (MatTek Corporation). Toxicity will be determined by measuring cytotoxicity [based on the relative conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)] in cultures treated with the test or control articles in the presence or absence of UVA/visible light.

Applications and Use⁹⁸

- The EpiDerm™ model is composed of human keratinocytes stratified into a 3D dermal structure consisting of basal, spinous, and granular layers, including a functioning *stratum corneum* with characteristic lipid lamellae.
- The test materials are applied directly to the culture surface, at air interface, so that undiluted and/or end-use dilutions can be tested directly.
- Ingredients for concentrations of formulations or final formulations (which cannot be assessed in the 3T3 NRU phototoxicity assay) can be tested using this model.
- Phototoxicity, photoirritancy, as well as photoprotective action (such as sunscreens) can be assessed.

Experimental Procedure**Sample and Control Preparations**

- The test articles are tested by topical application; dilutions are prepared the day of dosing.
- The water-soluble test articles are suspended in Hanks' balanced salt solution w/o phenol red (HBSS). Water-insoluble test articles are diluted in DMSO (minimal UV absorbance).
- The positive control, chlorpromazine, is diluted in DMSO (200X stock dilution concentration), and subsequently diluted to a final concentration of 0.02% (0.2 mg/mL) in HBSS.
- The negative control, 50 μ L of HBSS for water-soluble test articles, or 50 μ L of HBSS supplemented with 0.1% DMSO for water-insoluble articles, is used to measure cytotoxicity of the UV exposure and the cytotoxicity of the positive control and test articles.
- A solvent control is necessary if DMSO is used.

Receipt and Preparation of Cultures

- Each culture is removed with sterile forceps from the agarose gel, inspected, and transferred to a pre-labeled 6-well plate containing 0.9 mL of AM per well. The EpiDerm™ cultures are incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a humidified atmosphere of $5\% \pm 1\%$ CO_2 for at least 1 h. After 1 h, the AM is removed and replaced with fresh AM prior to dosing.

Assay Procedure

- 50 μ L of each dose is applied to each designated tissue.
- Four tissues for each test article concentration or control group are treated and incubated for up to 24 h at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $5\% \pm 1\%$ CO_2 . This period allows the test or control articles to penetrate the tissue construct.
- Tissues dosed with test articles that may interfere with the UVA exposure (i.e., creams), are rinsed w/ sterile D-PBS w/o Ca^{++} and Mg^{++} , and cotton tipped applicators may be used to remove the test article immediately prior to UVA or dark exposure.
- 24-well plates receive 0.3 mL of HBSS in each well. Each treatment group is divided into two UV exposure groups, UVA or dark. Two tissues from each group are transferred into the 24-well plates designated for UVA or dark exposure.
- The UVA plates are exposed (with the lid on) to 1.7 ± 0.1 mW/cm^2 UVA light (resulting in an irradiation dose of 6 J/cm^2), while the remaining dark plates are placed in the dark. Both exposures are for 60 ± 2 min at room temperature.
- After the UVA or dark exposure, the tissues are rinsed w/ sterile D-PBS w/o Ca^{++} and Mg^{++} , and placed back into the 6-well plates containing 0.9 mL of fresh, warmed AM containing penicillin/streptomycin. The 6-well plates are incubated for a 21 ± 1 h at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $5\% \pm 1\%$ CO_2 .
- After 21 h, the cultures are transferred to wells containing 0.3 mL of MTT reagent (1 mg/mL) and incubated for 3 h.
- After 3 h, the cultures are blotted on absorbent paper and extracted in 2 mL of isopropanol for 2 h, while shaking.
- 200 μ L of each extraction solution is transferred to a 96-well plate and the absorbance at 550 nm (OD_{550}) determined.

Data Evaluation

- The relative survival is determined by comparing the mean corrected OD_{550} value of the test article-treated wells to the mean corrected OD_{550} value of the negative control-treated wells.
- A 30% decrease in viability in treated cultures in the presence of UVA compared to the viability in the absence of UVA is predictive of phototoxic potential.

REFERENCES

1. Brantom, P., Bruner, L., Chamberlain, M., De Silva, O., Dupuis, J., Earl, L. K., Lovell, D. et al., A summary report of the COLIPA international validation study on alternatives to the Draize rabbit eye irritation test, *Toxicology In Vitro*, 11, 141–179, 1997.
2. Balls, M., Botham, P., Bruner, L., and Spielmann, H., The EC/HO international validation study on alternatives to the Draize eye irritation test, *Toxicology In Vitro*, 9, 871–929, 1995.
3. Harbell, J. W., Koontz, S. W., Lewis, R. W., Lovell, D., and Acosta, D., IRAG working group 4. Cell cytotoxicity assays. Interagency Regulatory Alternatives Group, *Food and Chemical Toxicology*, 35, 79–126, 1997.
4. Gettings, S. D., Teal, J. J., Bagley, D. M., Demetruilas, J., Dipasquale, L. C., Hintze, K. L., Rozen, M. G., Weisse, S. L., Chudkowski, M., Marenus, K. D., Pape, W., Roddy, M. T., Schnetzinger, R., Silber, P. M., Glaza, S. M., and Kurtz, P. J., The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize eye irritation test (phase I) hydro-alcoholic formulations; (part 2) data analysis and biological significance, *In Vitro Toxicology*, 4, 247–288, 1991.
5. Osborne, R. and Perkins, M. A., An approach for development of alternative test methods based on mechanisms of skin irritation, *Food and Chemical Toxicology*, 32, 133–142, 1994.
6. Osborne, R., Perkins, M. A., and Roberts, D. A., Development and intralaboratory evaluation of an *in vitro* human cell-based test to aid ocular irritancy assessments, *Fundamental and Applied Toxicology*, 28, 139–153, 1995.
7. Costin, G. E., Raabe, H., and Curren, R., *In vitro* safety testing strategy for skin irritation using the 3D reconstructed human epidermis, *Romanian Journal of Biochemistry*, 46, 165–186, 2009.
8. Freshney, R. I., *Culture of Animal Cells: A Manual of Basic Technique*. New York: Wiley-Liss, 2005.
9. Scott, L., Eskes, C., Hoffmann, S., Adriaens, E., Alepee, N., Bufo, M., Clothier, R. et al., Bottom-up and top-down approach: Eye irritation testing strategy to reduce and replace *in vivo* studies, *Toxicology In Vitro*, 24, 1–9, 2010.
10. Freeberg, F. E., Griffith, J. F., Bruce, R. D., and Bay, P. H. S., Correlation of animal test methods with human experience for household products, *Journal of Toxicology—Cutaneous and Ocular Toxicology*, 1, 53–64, 1984.
11. Griffith, J. F., Nixon, G. A., Bruce, R. D., Reer, P. J., and Bannan, E. A., Dose-response studies with chemical irritants in the albino rabbit eye as a basis for selecting optimum testing conditions for predicting hazard to the human eye, *Toxicology and Applied Pharmacology*, 55, 501–513, 1980.
12. Nussenblatt, R. B., Bron, A., Chambers, W. A., McCulley, J. P., Pericoi, M., Ubels, J. L., and Edelhauser, H. F., Ophthalmologic perspectives on eye irritation testing, *Journal of Toxicology—Cutaneous and Ocular Toxicology*, 17, 103–109, 1998.
13. Jester, J. V., Extent of corneal injury as a biomarker for hazard assessment and the development of alternative models to the Draize rabbit eye test, *Cutaneous and Ocular Toxicology*, 25, 41–54, 2006.
14. Sina, J. F. and Gautheron, P., Report from the Bovine Opacity and Permeability Assay Technical Workshop November 3–4, 1997. An historical perspective, *In Vitro & Molecular Biology*, 11, 316–326, 1998.
15. Curren, R., Southee, J. A., Spielmann, H., Liebsch, H. M., Fentem, J., and Balls, M., The role of prevalidation in the development, validation and acceptance of alternative methods, *Alternatives to Animal Experimentation*, 23, 211–217, 1995.
16. Bruner, L., Carr, G., Chamberlain, M., and Curren, R., Validation of alternative methods for toxicity testing, *Toxicology In Vitro*, 10, 479–501, 1996.
17. Bruner, L., Carr, G., Chamberlain, M., and Curren, R., No prediction model, no validation study, *Alternatives to Animal Experimentation*, 24, 139–142, 1996.
18. Cooper-Hannan, R., Harbell, J., Coecke, S., Balls, M., Bowe, G., Cervinka, M., Clothier, R., Hermann, F., Klahm, L. K., de Lange, J., Liebsch, H. M., and Vanparys, P., The principles of good laboratory practice: application to *in vitro* toxicology studies, *Alternatives to Laboratory Animals*, 27, 539–577, 1999.
19. Anonymous, ECVAM news and views, *Alternatives to Animal Experimentation*, 22, 7–11, 1994.
20. Anonymous, Validation and regulatory acceptance of toxicological test methods, National Institute of Health Publication 97–3981. In *Interagency Coordinating Committee on the Validation of Alternative Methods*. Research Triangle Park, NC: National Institute of Environmental Health Sciences, 1997.
21. Kojima, H., Ando, T., Inagaki, K., Ohhira, M., Kosaka, T., Nakamura, Y., Torishima, H. et al., Validation of human skin models for skin corrosivity tests in Japan, *Alternatives to Animal Experimentation*, 13, 36–44, 2008.
22. Stokes, W. and Wind, M., The international cooperation on alternative test methods (ICATM): translating science to provide improved public health safety assessment tools, *The Toxicologist*, 120, 89, 2011.
23. OECD, Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants: OECD Guideline for Testing of Chemicals. No. 437, Organization for Economic Cooperation and Development, 2009.
24. OECD, Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants: OECD Guideline for Testing of Chemicals. No. 438, Organization for Economic Cooperation and Development, 2009.
25. OECD, *In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test (TER): OECD Guideline for Testing of Chemicals. No. 430, Organization for Economic Cooperation and Development, 2004.
26. OECD, *In Vitro* Skin Corrosion: Human Skin Model Test: OECD Guideline for Testing of Chemicals. No. 431, Organization for Economic Cooperation and Development, 2004.
27. OECD, *In Vitro* Membrane Barrier Test Method for Skin Corrosion: OECD Guideline for Testing of Chemicals. No. 435, Organization for Economic Cooperation and Development, 2006.
28. OECD, *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method: OECD Guideline for Testing of Chemicals. No. 439, Organization for Economic Cooperation and Development, 2010.
29. Ahmad, A. E., Aronson, J., and Jacobs, S., Induction of oxidative stress and TNF-alpha secretion by dichloroacetonitrile, a water disinfectant by-product, as possible mediators of apoptosis or necrosis in a murine macrophage cell line (RAW), *Toxicology In Vitro*, 14, 199–210, 2000.
30. Nieminen, A. L., Gores, G. J., Bond, J. M., Imberti, R., Herman, B., and Lemasters, J. J., A novel cytotoxicity screening assay using a multiwell fluorescent scanner, *Toxicology and Applied Pharmacology*, 115, 145–155, 1992.
31. Rhoads, L. S., Cook, J. R., Patrone, L. M., and van Buskirk, R. G., A human epidermal model can be assayed employing a multiple fluorescent endpoint assay and the Cytofluor 2300, *Journal of Toxicology—Cutaneous and Ocular Toxicology*, 12, 87–108, 1993.

32. Magoffin, D. A., Kushell, D. L., and Schlesinger, R. J., The chromium release cytotoxicity test: comparison to the agar overlay and cell-growth inhibition tests. In *Cell-Culture Test Methods, ASTM STP 810*. Brown, S.A. Ed., American Society for Testing and Materials, 1983.
33. Bockman, C. S., Griffith, M., and Watcky, M. A., Properties of whole-cell ionic currents in cultured human corneal epithelial cells, *Investigative Ophthalmology and Vision Sciences*, 39, 1143–1151, 1998.
34. Buckman, S. Y., Gresham, M., Hale, P., Hruza, G., Anast, J., Masferrer, J., and Pentland, A. P., COX-2 expression is induced by UVB exposure in human cells: implications for the development of skin cancer, *Carcinogenesis* 19, 723–729, 1998.
35. McConnell, H. M., Owicki, J. C., Parce, J. W., Miller, D. L., Baxter, G. T., Wada, H. G., and Pitchford, S., The Cytosensor microphysiometer: Biological applications of silicon technology, *Science*, 257, 1906–1912, 1992.
36. Harbell, J. W., Osborne, R., Carr, G., and Peterson, A., Assessment of the cytosensor microphysiometer assay in the COLIPA in vitro eye irritation validation study *Toxicology In Vitro*, 13, 313–323, 1999.
37. Masters, B. A., Palmoski, M. J., Flint, O. P., Gregg, R. E., and Wang-Iverson, D., In vitro myotoxicity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, Pravastatin, Lovastatin, and Simvastatin, *Toxicology and Applied Pharmacology*, 131(1), 163–174, 1995.
38. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *Journal of Immunological Methods*, 65, 55–63, 1983.
39. Weislow, O. S., Kiser, R., Fine, D. L., Bader, J., Shoemaker, R. H., and Boyd, M. R., New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity, *Journal of the National Cancer Institute*, 81, 577–586, 1989.
40. Filman, D. J., Brawn, R. J., and Dandliker, W. B., Intracellular supravital stain delocalization as an assay for antibody-dependent complement-mediated cell damage, *Journal Of Immunological Methods*, 6, 189–207, 1975.
41. Barstad, R., Cortesi, J., and Janus, J., Use of clonetics neutral red bioassay to optimize components of serumfree medium for normal human anchorage-dependent cells, *In Vitro Cell and Developmental Biology*, 27, 160, 1991.
42. Jiang, T., Grant, R. L., and Acosta, D., A digitized fluorescence imaging study of intracellular free calcium, mitochondrial integrity and cytotoxicity in rat renal cells exposed to ionomycin. A calcium ionophore, *Toxicology*, 85, 41–65, 1993.
43. Jester, J. V., Barry, P. A., Lind, G. L., Petroll, W. M., Garana, R., and Cavanagh, H. D., Corneal keratocytes: in situ and in vitro organization of cytoskeletal contractile proteins, *Investigative Ophthalmology & Visual Science*, 39, 922–936, 1994.
44. North-Root, H., Yackovich, F., Demetruilis, J., Gacula, M., Jr., and Heinze, J. E., Evaluation of an in vitro cell toxicity test using rabbit corneal cells to predict the eye irritation potential of surfactants. In *Safety Evaluation and Regulation of Chemicals*. Homberger, F. Ed., Krager, Basel, Switzerland, 1983, pp. 259–269.
45. Pham, X. T. and Huff, J. W., Cytotoxic evaluation of multi-purpose contact lens solutions using an in vitro test battery, *Contact Lens Association of Ophthalmologists Journal*, 25, 28–35, 1999.
46. Livingston, R. B., Titus, G. A., and Heilbrun, L. K., In vitro effects on DNA synthesis as a predictor of biological effect from chemotherapy, *Cancer Research*, 40, 2209–2212, 1980.
47. Harbell, J. W., Wallace, K. A., Curren, R. D., Naughton, G. K., and Triglia, D., A comparison of four measures of toxicity applied to human dermal fibroblasts grown the three dimensional culture on nylon mesh (Skin2 Dermal Model). In *Alternative Methods in Toxicology*. Goldberg, A. Ed., Mary Ann Liebert, New York, 1991, Vol. 8, pp. 301–309.
48. Lewis, R. W., McCall, J. C., and Botham, P., A comparison of two cytotoxicity tests for predicting the ocular irritancy of surfactants, *Toxicology In Vitro*, 7, 155–158, 1993.
49. Berridge, M. V., Tan, A. S., McCoy, K. D., and Wang, R., The biochemical and cellular basis of cell proliferation assay that use tetrazolium salts, *Biochemica*, 4, 14–19, 1996.
50. Marshall, N. J., Goodwin, C. J., and Holt, S. J., A critical review of the use of microculture tetrazolium assays to measure cell growth and function, *Growth Regulation*, 5, 69–84, 1995.
51. Borenfreund, E. and Puerner, J. A., Toxicity determined in vitro by morphological alterations and neutral red absorption, *Toxicology Letters*, 24, 119–124, 1985.
52. Reader, S. J., Blackwell, V., O'Hara, R., Clothier, R., Griffin, G., and Balls, M., A vital dye release method for assessing the short term cytotoxic effects of chemicals and formulations, *Alternatives to Animal Experimentation*, 17, 28–37, 1989.
53. Harbell, J. W., Southee, J. A., and Curren, R., The path to regulatory acceptance of in vitro methods is paved with the strictest standards. In *Animal Alternatives, Welfare and Ethics*. van Zutphen, L.F.M. and Balls, M. Eds., Elsevier Science, Amsterdam, The Netherlands, 1997, pp. 1177–1181.
54. CDC, *Biosafety in Microbiological and Biomedical Laboratories*, U. S. Government Printing Office, Washington, DC, 1993.
55. Jester, J. V., Li, L., Molai, A., and Maurer, J. K., Extent of initial corneal injury as a basis for alternative eye irritation tests, *Toxicology In Vitro*, 15, 115–130, 2001.
56. Maurer, J. K., Li, H. F., Petroll, W. M., Parker, R. D., Cavanagh, H. D., and Jester, J. V., Confocal microscopic characterization of initial corneal changes of surfactant-induced eye irritation in the rabbit, *Toxicology and Applied Pharmacology*, 143, 291–300, 1997.
57. Maurer, J. K., Parker, R. D., and Jester, J. V., Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays, *Regulatory Toxicology and Pharmacology*, 36, 106–117, 2002.
58. Maurer, J. K., Molai, A., Parker, R. D., Li, L., Carr, G. J., Petroll, W. M., Cavanagh, H. D., and Jester, J. V., Pathology of ocular irritation with bleaching agents in the rabbit low-volume eye test, *Toxicologic Pathology*, 29, 308–319, 2001.
59. Curren, R., Evans, M., Raabe, H., Ruppalt, R., and Harbell, J., An histological analysis of damage to bovine corneas in vitro by selected ocular toxicants, *The Toxicologist*, 54, 188, 2000.
60. Harbell, J., Mun, G., and Curren, R., Application of histological evaluation to enhance the bovine corneal opacity and permeability (BCOP) assay, *The Toxicologist*, 90, 326, 2006.
61. Jester, J. V., Ling, J., and Harbell, J., Measuring depth of injury (DOI) in an isolated rabbit eye irritation test (IRE) using biomarkers of cell death and viability, *Toxicology In Vitro*, 24, 597–604, 2010.
62. Xu, K. P., Li, X. F., and Yu, F. S., Corneal organ culture model for assessing epithelial responses to surfactants, *Toxicological Sciences*, 58, 306–314, 2000.
63. Piehl, M., Carathers, M., Soda, R., Cerven, D., and DeGeorge, G., Porcine Corneal Ocular Reversibility Assay (PORCORA) predicts ocular damage and recovery for global regulatory agency hazard categories, *Toxicology In Vitro*, 25, 1912–1918, 2011.

64. Gautheron, P., Dukic, M., Alix, D., and Sina, J. F., Bovine corneal opacity and permeability test: an in vitro assay of ocular irritancy, *Fundamental and Applied Toxicology*, 18, 442–449, 1992.
65. Sina, J. F., Galer, D. M., Sussman, R. G., Gautheron, P. D., Sargent, E. V., Leong, B., Shah, P. V., Curren, R. D., and Miller, K., A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates, *Fundamental and Applied Toxicology*, 26, 20–31, 1995.
66. Bagley, D. M., Cervin, D., and Harbell, J. W., Assessment of the chorioallantoic membrane vascular assay (CAMVA) in the COLIPA in vitro eye irritation validation study, *Toxicology In Vitro*, 13, 285–293, 1999.
67. Bagley, D. M., Waters, D., and Kong, B. M., Development of a 10-day chorioallantoic membrane vascular assay as an alternative to the Draize eye irritation test, *Food and Chemical Toxicology*, 32, 1155–1160, 1994.
68. Chamberlain, M., Gad, S. C., Gautheron, P., and Prinsen, M. K., IRAG working group 1. Organotypic models for the assessment/prediction of ocular irritation. Interagency Regulatory Alternatives Group, *Food and Chemical Toxicology*, 35, 23–37, 1997.
69. Tchao, R., Trans-epithelial permeability of fluorescein in vitro as an assay to determine eye irritants. In *Alternative Methods in Toxicology*. Goldberg, A.M. Ed., Mary Ann Liebert, Vol. 6, pp. 271–283, 1988.
70. Shaw, A. J., Clothier, R., and Balls, M., Loss of trans-epithelial impermeability of a confluent monolayer of Madin-Darby canine kidney (MDCK) cells as a determinant of ocular irritancy potential, *Alternatives to Animal Experimentation*, 18, 145–151, 1990.
71. Kruszewski, F. H., Walker, T. L., and DiPasquale, L. C., Evaluation of a human corneal epithelial cell line as an in vitro model for assessing ocular irritation, *Fundamental and Applied Toxicology*, 36, 130–140, 1997.
72. Luepke, N. P. and Kemper, F. H., HET-CAM: as alternative to the Draize eye test, *Food and Chemical Toxicology*, 24, 495–496, 1986.
73. Spielmann, H., Liebsch, M., Moldenhauer, F., Holzhütter, H. G., Bagley, D. M., Lipman, J. M., Pape, W. J. et al., IRAG working group 2. CAM-based assays. Interagency Regulatory Alternatives Group, *Food And Chemical Toxicology: An International Journal Published For The British Industrial Biological Research Association*, 35, 39–66, 1997.
74. Zuang, V., The neutral red release assay: A review, *Alternatives to Animal Experimentation*, 29, 575–599, 2001.
75. Pape, W., Pfannenbecker, U., and Hoppe, U., Validation of the red blood cell test system as an in vitro assay for the rapid screening of irritation potential of surfactants, *Molecular Toxicology*, 1, 525–536, 1987.
76. Takahashi, Y., Koike, M., Honda, H., Ito, Y., Sakaguchi, H., Suzuki, H., and Nishiyama, N., Development of the short time exposure (STE) test: An in vitro eye irritation test using SIRC cells, *Toxicology In Vitro*, 22, 760–770, 2008.
77. Blazka, M. E., Harbell, J., Klausner, M., Raabe, H., Kubilus, J., Hsia, F., Minerath, B., Kotler, M., and Bagley, D. M., Colgate-Palmolive's program to validate the EpiOcular human tissue construct model, *The Toxicologist*, 54, 188, 2000.
78. Van Goethem, F., Adriaens, E., Alepee, N., Straube, F., De Wever, B., Cappadoro, M., Catoire, S., Hansen, E., Wolf, A., and Vanparys, P., Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals, *Toxicology In Vitro*, 20, 1–17, 2006.
79. Kaluzhny, Y., Kandarova, H., Hayden, P., Kubilus, J., d'Argembeau-Thornton, L., and Klausner, M., Development of the EpiOcular eye irritation test for hazard identification and labeling of eye irritating chemicals in response to the requirements of the EU cosmetics directive and REACH legislation, *Alternatives to Animal Experimentation*, 39, 339–364, 2011.
80. Bagley, D. M., Booman, K. A., Bruner, L., Casterton, P. L., Demetrulias, J., Heinze, J. E., Innis, J. D., McCormick, W. C., Neun, A. S., Rothenstein, A., and Sedlak, R. I., The SDA program phase III: comparison of in vitro data with animal eye irritation data on solvents, surfactants, oxidizing agents, and prototype cleaning products, *Journal of Toxicology—Cutaneous and Ocular Toxicology*, 13, 127–155, 1994.
81. Takahashi, H., Hayashi, K., Abo, T., Koike, M., Sakaguchi, H., and Nishiyama, N., The Short Time Exposure (STE) test for predicting eye irritation potential: intra-laboratory reproducibility and correspondence to globally harmonized system (GHS) and EU eye irritation classification for 109 chemicals, *Toxicology In Vitro*, 25, 1425–1434, 2011.
82. Sakaguchi, H., Ota, N., Omori, T., Kuwahara, H., Sozu, T., Takagi, Y., Takahashi, H., Tanigawa, K., Nakanishi, M., Nakamura, T., Morimoto, T., Wakuri, S., Okamoto, Y., Sakaguchi, H., Hayashi, T., Hanji, T., and Watanabe, S., Validation study of the Short Time Exposure (STE) test to assess the eye irritation potential of chemicals, *Toxicology In Vitro*, 25, 796–809, 2011.
83. Harbell, J. and Curren, R., The bovine corneal opacity and permeability assay: observations on assay performance, *In Vitro Molecular Toxicology*, 11, 337–341, 1998.
84. Harbell, J. W., Raabe, H., Dobson, T., Evans, M., and Curren, R., Histopathology associated with opacity and permeability changes in the bovine corneas in vitro, *Alternatives to Animal Experimentation*, 27, 347, 1999.
85. Cater, K. C. and Harbell, J. W., Prediction of eye irritation potential of surfactant-based rinse-off personal care formulations by the bovine corneal opacity and permeability (BCOP) assay, *Cutaneous and Ocular Toxicology*, 25, 217–233, 2006.
86. Gettings, S. D., Lordo, R. A., Hintze, K. L., Bagley, D. M., Casterton, P. L., Chudkowski, M., Curren, R. et al., The CTFA evaluation of alternatives program: An evaluation of in vitro alternatives to the Draize primary eye irritation test (Phase III). Surfactant-based formulations, *Food and Chemical Toxicology*, 34, 79–117, 1996.
87. Schrage, A., Kolle, S. N., Moreno, M. C. R., Norman, K., Raabe, H., Curren, R., Van Ravenzwaay, B., and Landsiedel, R., The bovine corneal opacity and permeability test in the routine ocular irritation testing and its improvement within the limits of the OECD Test Guideline 437, *Alternatives to Laboratory Animals*, 39, 37–53, 2011.
88. Harbell, J. and Curren, R., Report from the Bovine Opacity and Permeability Technical Workshop November 3–4, 1997. The bovine opacity and permeability assay: Observations on assay Performance, *In Vitro & Molecular Biology*, 11, 337–341, 1998.
89. Stern, M., Klausner, M., Alvarado, R., Rensker, K., and Dickens, M., Evaluation of the EpiOcular tissue model as an alternative to the Draize eye irritation test, *Toxicology In Vitro*, 12, 445–461, 1998.
90. Blazka, M. E., Harbell, J., Klausner, M., Merrill, J., Kubilus, J., Kloss, C., and Bagley, D. M., Evaluating the ocular irritation potential of 54 test articles using the EpiOcular human tissue construct model (OCL-200), *The Toxicologist*, 72, 221, 2003.

91. Eskes, C., Bessou, S., Bruner, L., Curren, R., Harbell, J., Jones, P., Kreiling, R., Liebsch, M., McNamee, P., Pape, W., Prinsen, M. K., Seidle, T., Vanparys, P., Worth, A., and Zuang, V., Eye irritation, *Alternatives to Laboratory Animals*, 33(Suppl. 1), 47–81, 2005.
92. McCain, N. E., Binetti, R. R., Gettings, S. D., and Jones, B. C., Assessment of ocular irritation ranges of market-leading cosmetic and personal-care products using an in vitro tissue equivalent, *The Toxicologist*, 66, 243, 2002.
93. Bouwstra, J., Groenink, H. W., Kempenaar, J., Romeijn, S. C., and Poncet, M., Water distribution and natural moisturizer factor content in human skin equivalents are regulated by environmental relative humidity, *Journal of Investigative Dermatology*, 128, 378–388, 2008.
94. Gibbs, S., Vicanova, J., Bouwstra, J., Valstar, D., Kempenaar, J., and Poncet, M., Culture of reconstructed epidermis in a defined medium at 33 degrees C shows a delayed epidermal maturation, prolonged lifespan and improved stratum corneum, *Archives of Dermatological Research*, 289, 585–595, 1997.
95. Gibbs, S., Vietsch, H., Meier, U., and Poncet, M., Effect of skin barrier competence on SLS and water-induced IL-1 α expression, *Experimental Dermatology*, 11, 217–223, 2002.
96. Netzlaff, F., Lehr, C. M., Wertz, P. W., and Schaefer, U. F., The human epidermis model EpiSkin, SkinEthic and EpiDerm: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport, *European Journal of Pharmaceutics and Biopharmaceutics*, 60, 167–178, 2005.
97. OECD, In Vitro 3T3 NRU Phototoxicity Test: OECD Guideline for Testing of Chemicals. No. 432, Organization for Economic Cooperation and Development, 2004.
98. Liebsch, H. M., Traue, D., Barrabas, C., Spielmann, H., Gerberick, G. F., Cruse, L. W., Diembeck, W. et al., Prevalidation of the EpiDerm phototoxicity test. In *Alternatives to Animal Testing II*. Clark, D.G., Lisansky, S.G., and Macmillan, R. Eds., CPL Press, Newbury, Berkshire, UK, 1999, pp. 160–167.
99. Liebsch, M., Spielmann, H., Pape, W., Krul, C., Deguercy, A., and Eskes, C., UV-induced effects, *Alternatives to Laboratory Animals*, 33(Suppl. 1), 131–146, 2005.
100. Wallace, K. A., Harbell, J., Accomando, N., Valone, S., and Curren, R., An evaluation of the human epidermal keratinocytes neutral red release and neutral red uptake assay using the first 10 MEIC test materials, *Toxicology In Vitro*, 6, 367–371, 1992.
101. Wilmer, J. L., Bureson, F. G., Kayama, F., Kanno, J., and Luster, M. I., Cytokine induction in human epidermal keratinocytes exposed to contact irritants and its relation to chemical-induced inflammation in mouse skin, *Journal of Investigative Dermatology*, 102, 915–922, 1994.
102. Harrop, A. R., Ghahary, A., Scott, P. G., Forsyth, N., Ujj-Friedland, R. T. A., and Tredget, E. E., Regulation of collagen synthesis and mRNA expression in normal and hypertrophic scar fibroblasts in vitro by interferon-gamma, *Journal of Surgical Research*, 58, 471–477, 1995.
103. Aberdam, E., Romero, C., and Ortonne, J. P., Repeated UVB irradiations do not have the same potential to promote stimulation of melanogenesis in cultured normal human melanocytes, *Journal of Cell Science*, 106, 1015–1022, 1993.
104. Detmar, M., Tenorio, S., Hettmannsperger, U., Ruszczak, Z., and Orfanos, C. E., Cytokine regulation of proliferation and ICAM-1 expression of human dermal microvascular endothelial cells in vitro, *Journal of Investigative Dermatology*, 98, 147–153, 1992.
105. Alba, S., Terunuma, A., Monome, H., and Tagami, H., Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules, *European Journal of Immunology*, 27, 3031–3038, 2005.
106. Spielmann, H., Balls, M., Dupuis, J., Pape, W., Pechovitch, G., De Silva, O., Holzhutter, H. et al., The international EU/COLIPA in vitro phototoxicity validation study: Results of phase II (blind trial); part 1: The 3T3 NRU phototoxicity test, *Toxicology In Vitro*, 12, 305–327, 1998.
107. Holzhutter, H., A general measure of in vitro phototoxicity derived from pairs of dose-response curves and its use for predicting in vitro phototoxicity of chemicals, *Alternatives to Laboratory Animals*, 25, 445–462, 1997.
108. Spielmann, H., Balls, M., Dupuis, J., Pape, W., De Silva, O., Holzhutter, H., Gerberick, G. F., Liebsch, H. M., Lovell, W. W., and Pfannenbecker, U., A study on UV filter chemicals from Annex VII of European Union Directive 76/768/EEC, in the in vitro 3T3 NRU phototoxicity test, *Alternatives to Laboratory Animals*, 26, 679–708, 1998.
109. Zerler, B., Roemer, E., Raabe, H., Reeves, A., and Harbell, J. W., Evaluation of the phototoxic potential of chemically modified tetracyclines using the 3T3 Neutral Red Assay. In *Progress in the Reduction, Refinement and Replacement of Animal Experimentation*. Balls, M., van Zeller, A.M., and Halder, M.E. Eds., Elsevier Sciences, Amsterdam, The Netherlands, 2000, Vol. 27, pp. 545–554.
110. Bronaugh, R. L. and Collier, S. W., In vitro methods for measuring skin penetration. In *Skin Penetration Fundamentals and Applications*. Zatz, J.L. Ed., Allured Publishing Corporation, 1993, pp. 93–111.
111. Hoffmann, J., Heister, E., Karpinski, S., Loose, J., Thomas, D., Siefken, W., Ahr, H. J., Wohr, H. W., and Fuchs, H. W., Epidermal-skin-test 1000 (EST-1000) a new reconstructed epidermis for in vitro skin corrosivity testing, *Toxicology In Vitro*, 19, 925–929, 2005.
112. Kandarova, H., Hayden, P., Klausner, M., Kubilus, J., Kearney, P., and Sheasgreen, J., In vitro skin irritation testing: improving the sensitivity of the EpiDerm skin model irritation test protocol, *Alternatives to Laboratory Animals*, 37, 671–689, 2009.
113. Roguet, R., Cohen, C., Robles, C., Courtellemont, P., Tolle, M., Guillot, J. P., and Pouradier Duteil, X., An interlaboratory study of the reproducibility and relevance of EpiSkin, a reconstructed human epidermis, in the assessment of cosmetic irritancy, *Toxicology In Vitro*, 12, 295–304, 1998.
114. Osborne, S., Mayer, F. K., Spake, A., Rosdy, M., De Wever, B., Ettlin, R. A., and Cordier, A., Predictivity of an in vitro model for acute and chronic skin irritation (SkinEthic) applied to the testing of topical vehicles, *Cell Biology and Toxicology*, 15, 121–135, 1999.
115. Rasmussen, C., Gratz, K., Liebel, F., Southall, M., Garay, M., Bharracharya, S., Simon, N., Vander Zanden, M., Van Winkle, K., Pirnstill, J., Pirnstill, S., Comer, A., and Allen-Hoffmann, B. L., The StrataTest human skin model, a consistent in vitro alternative for toxicological testing, *Toxicology In Vitro*, 24, 2021–2029, 2010.
116. Kojima, H., Ando, Y., Idehara, M., Kosaka, T., Miyalka, E., Shinoda, S., Suzuki, T., Yamaguchi, Y., Yoshimura, I., Yuasa, A., Watanabe, Y., and Omori, T., Validation study of the in vitro skin irritation test with the LabCyte EPI-MODEL24, *Alternatives to Laboratory Animals*, 40, 33–50, 2012.

117. Liebsch, H. M., Traue, D., Barrabas, C., Spielmann, H., Uphill, P., Wilkins, S., McPherson, J. P., Wiemann, C., Kaufmann, T., Remmele, M., and Holzhuetter, H. G., The ECVAM prevalidation study on the use of EpiDerm for skin corrosivity testing, *Alternatives to Animal Experimentation*, 28, 371–401, 2000.
118. Fentem, J. H., Briggs, D., Chesne, C., Elliott, G. R., Harbell, J. W., Heylings, J. R., Portes, P., Roguet, R., van de Sandt, J. J., and Botham, P. A., A prevalidation study on in vitro tests for acute skin irritation: Results and evaluation by the Management Team, *Toxicol In Vitro*, 15, 57–93, 2001.
119. Koschier, F. J., Roth, R. N., Wallace, K. A., Curren, R., and Harbell, J., A comparison of three dimensional human skin models to evaluate the dermal irritation of selected petroleum products, *In Vitro Toxicology*, 10, 391–406, 1997.

6 Inhalation Toxicology*

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INTRODUCTION

Potential exposure to toxic materials is greater via inhalation than any other route. This potential is due to the large lung surface area available for gas transport (~90 m² in an adult human) and the relative ease of passage from alveolus to blood for most substances. Some inhalation exposures may be intentional, as with inhaled drugs. However, many exposures are unintentional via environmental pollutants in the industrial setting or ambient air. Inhalation toxicity studies determine the health effects of these materials through exposure to animals, which then allows for human risk assessment. Inhalation studies involve all the

standard types of toxicity studies and their various end points including acute, subchronic, chronic, oncogenicity, reproductive, developmental, neurotoxicity, as well as safety pharmacology and *in vitro* exposures. The following tables and graphs are a compilation that has proved to be useful in the conduct of inhalation studies and the extrapolation of effects among different species. The compilation includes data on respiratory tract anatomy, pulmonary function, bronchoalveolar lavage, metabolism, pulmonary deposition and clearance, pulmonary toxicity, and data associated with exposure chambers and the generation and monitoring of exposure atmospheres.

* In memory of Paul E. Newton, PhD, DABT.

RESPIRATORY TRACT ANATOMY (TABLES 6.1 THROUGH 6.12; FIGURES 6.1 THROUGH 6.7)

TABLE 6.1

Comparative Lung Biology: Morphologic Features of Pleura, Interlobular, and Segmental Septa, and Distal Airways

	Human	Macaque Monkey	Dog, Cat	Ferret	Mouse, Rat, Gerbil, Hamster, Guinea Pig, Rabbit	Horse, Sheep	Ox, Pig
Pleura	Thick	Thin	Thin	Thin	Thin	Thick	Thick
Interlobular and segmental connective tissue	Extensive, interlobular partially surrounds many lobules	Little	Little, if any	Little	Little, if any	Extensive ^a interlobular partially surrounds many lobules	Extensive, interlobular surrounds completely
Nonrespiratory bronchiole (nonalveolarized)	Several generations	Fewer generations, commonly only one	Fewer generations	Several generations	Several generations	Several generations	Several generations
	TB ends in respiratory bronchioles	TB ends in respiratory bronchioles	TB ends in respiratory bronchioles	TB ends in respiratory bronchioles	TB ends in alveolar ducts or very short respiratory bronchioles	TB ends in alveolar ducts or very short respiratory bronchioles	TB ends in alveolar ducts or very short respiratory bronchioles
Respiratory bronchiole (alveolarized)	Several generations	Several generations	Several generations	Several generations	Absent or a single short generation	Absent or a single short generation	Absent or a single short generation

Source: Tyler, W.S. and Julian, M.D., Gross and subgross anatomy of lungs, pleura, connective tissue septa, distal airways and structural units, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: TB, terminal nonrespiratory bronchiole.

^a The interlobular connective tissue of the sheep appears extensive, and lobules appear completely separated in gross preparations, but not in LM, SEM, or HRCT.

TABLE 6.2

Interspecies Comparison of Nasal Cavity Characteristics

	Sprague-Dawley Rat	Guinea Pig	Beagle Dog	Rhesus Monkey	Man
Body weight	250 g	600 g	10 kg	7 kg	~70 kg
Naris cross section	0.7 mm ²	2.5 mm ²	16.7 mm ²	22.9 mm ²	140 mm ²
Bend in naris	40°	40°	30°	30°	
Length	23 cm	3.4 cm	10 cm	5.3 cm	7–8 cm
Greatest vertical diameter	9.6 mm	12.8 mm	23 mm	27 mm	40–45 mm
Surface area (both sides of nasal cavity)	10.4 cm ²	27.4 cm ²	220.7 cm ²	61.6 cm ²	181 cm ²
Volume (both sides)	0.4 cm ³	0.9 cm ³	20 cm ³	8 cm ³	16–19 cm ³ (does not include sinuses)
Bend in nasopharynx	15°	30°	30°	80°	~90°
Turbinate complexity	Complex scroll	Complex scroll	Very complex membranous	Simple scroll	Simple scroll

Source: Schreider, J.P., Comparative anatomy and function of the nasal passages, in *Nasal Tumors in Animals and Man, Vol. III, Experimental Nasal Carcinogenesis*, Barrow, C.S., Ed., CRC Press, Boca Raton, FL, 1983. With permission.

TABLE 6.3
Comparative Anatomy of the Lung Parenchyma and AirBlood Tissue Barrier^a

Species	N	Body Weight (g)	Lung Volume (mL)	Alveolar Surface Area (Both lungs), cm ²	Capillary Surface Area (Both lungs), cm ²	Capillary Volume (Both Lungs), mL	Tissue (μm)
Shrew (<i>Sarcus etruscus</i>)	4	2.6 ± 0.2	0.10 ± 0.01	170 ± 10	130 ± 15	0.0118 ± 0.002	0.27 ± 0.02
White mouse (<i>Mus musculus</i>)	5	23 ± 2	0.74 ± 0.07	680 ± 85	590 ± 60	0.084 ± 0.009	0.32 ± 0.01
Waltzing mouse (<i>Mus wagneri</i>)	5	13 ± 1	0.58 ± 0.06	630 ± 40	540 ± 30	0.065 ± 0.008	0.26 ± 0.002
Syrian golden hamster (<i>Mesocricetus auratus</i>)	4	118 ± 7	2.81 ± 0.24	2,760 ± 250	2,410 ± 190	0.294 ± 0.011	0.39 ± 0.10
White rat (<i>Rattus rattus</i>)	8	140 ± 7	6.34 ± 0.25	3,880 ± 190	4,070 ± 200	0.480 ± 0.022	0.37 ± 0.02
White rat (Sprague-Dawley)	6	360 ± 4	10.82 ± 0.38	4,865 ± 380	4,270 ± 385	0.63 ± 0.07	0.40 ± 0.02
White rat (Fischer-344)							
Male: 5 months	4	289 ± 13	8.60 ± 0.31	3,915 ± 390	3,830 ± 395	0.65 ± 0.06	0.38 ± 0.03
Female: 5 months	4	182 ± 5	7.48 ± 0.10	3,420 ± 125	3,260 ± 185	0.46 ± 0.10	0.34 ± 0.01
Male: 26 months	4	391 ± 11	12.67 ± 0.74	4,630 ± 440	4,490 ± 485	0.67 ± 0.10	0.37 ± 0.01
Female: 26 months	4	298 ± 7	9.39 ± 0.40	4,020 ± 25	3,570 ± 165	0.34 ± 0.05	0.37 ± 0.01
Guinea pig (<i>Cavia porcellus</i>)	15	429 ± 11	13.04 ± 3.03	9,100 ± 280	7,400 ± 230	1.50 ± 0.08	0.42 ± 0.01
Rabbit (<i>Oryctolagus cuniculus</i>)	6	3,560	79.2	58,600 ± 12,400	47,000 ± 8,800	7.15 ± 1.88	0.50 ± 0.04
Dwarf mongoose (<i>Helogale parvula</i>)	3	52,800 ± 9,800	30.6 ± 5.6	16,100 ± 2,600	14,600 ± 3,400	2.06 ± 0.52	0.39 ± 0.02
Genet cat (<i>Genetta tigrina</i>)	2	137,200 ± 4,300	99.0 ± 12.2	56,300 ± 6,400	42,300 ± 1,600	5.04 ± 0.63	0.51 ± 0.02
Dog (<i>Canis familiaris</i>)	3	5,400	284.2	182,000 ± 135,000	141,000 ± 111,000	26.0 ± 24.9	0.43 ± 0.02
Dog (<i>C. familiaris</i>)	8	11,200 ± 400	736 ± 25	407,000 ± 39,000	329,000 ± 16,000	50.2 ± 5.0	0.46 ± 0.01
Dog (<i>C. familiaris</i>)	4	16,000 ± 3,000	1,322 ± 64	510,000 ± 10,000	570,000 ± 20,000	92 ± 5	0.45 ± 0.01
Dog (<i>C. familiaris</i>)	6	22,800 ± 600	1,501 ± 74	897,000 ± 69,000	718,000 ± 69,000	71.8 ± 4.5	0.48 ± 0.01
Dog (<i>C. familiaris</i>)	5	46,100	2,888	1,769,000 ± 456,000	1,319,000 ± 375,000	234 ± 69	0.53 ± 0.08
Camel (<i>Camelus dromedarius</i>)	2	231,700 ± 2,700	15,900 ± 1,400	4,305,000 ± 584,000	2,726,000 ± 292,000	378 ± 100	0.60 ± 0.06
Giraffe (<i>Giraffa camelopardalis</i>)	1	383,000	21,000	6,361,000	5,516,000	965	0.60
Suni (<i>Nesotragus moschatus</i>)	2	3,300 ± 300	209.4 ± 0.6	96,900 ± 5,500	81,300 ± 13,000	12.4 ± 0.7	0.56 ± 0.09
Dik-dik (<i>Madoqua kirkii</i>)	2	4,200 ± 100	313.4 ± 1.2	146,000 ± 700	130,000 ± 6550	22.6 ± 3.3	0.43 ± 0.02
Wildebeest (<i>Connochaetes taurinus</i>)	1	102,000	7,678	3,908,000	2,813,000	472	0.37
Waterbuck (<i>Kobus defassa</i>)	2	109,800 ± 16,300	7,835 ± 1,550	3,829,000 ± 950,000	3,378,000 ± 460,000	584 ± 98	0.46 ± 0.04
African goat (<i>Capra hircus</i>)	2	20,900 ± 1,000	1,370 ± 15	449,000 ± 12,000	439,000 ± 12,000	101 ± 8	0.54 ± 0.03
African sheep (<i>Ovis aries</i>)	2	21,800 ± 200	17,055 ± 435	671,000 ± 71,000	645,000 ± 139,000	146 ± 35	0.53 ± 0.05
Zebu cattle (<i>Bos indicus</i>)	4	192,500 ± 24,000	10,145 ± 1,960	3,850,000 ± 420,000	3,795,000 ± 392,000	700 ± 124	0.50 ± 0.04
Swiss cow (<i>B. taurus</i>)	1	700,000	22,450	12,830,000	11,380,000	2,770	0.51
Horse (<i>Equus caballus</i>)	2	510,000 ± 0	37,650 ± 1,050	24,560,000 ± 124,000	16,630,000 ± 1,080,000	2,800 ± 300	0.60 ± 0.02
Monkey (<i>Macaca irus</i>)	6	3,710	184.2	133,000 ± 12,700	116,000 ± 15,400	15.5 ± 2.7	0.50 ± 0.03
Baboon (<i>Papio papio</i>)	5	29,000 ± 3,000	2,393 ± 100	496,000 ± 77,000	386,000 ± 95,000	44 ± 17	0.67 ± 0.06
Man (<i>Homo sapiens</i>)	8	74,000 ± 4,000	4,341 ± 285	1,430,000 ± 120,000	1,260,000 ± 120,000	213 ± 31	0.62 ± 0.04

Source: Pinkerton, R.E. et al., Architecture and cellular composition of the airblood barrier, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1991. With permission.
^a All values are mean ± SEM.

TABLE 6.4
Allometry of Pulmonary Structural Variables

Taxon	Intercept	Slope	N	M_b Range
V_L, mL; Lung Volume				
Mammals	56.7	1.02	21	0.01–2.000
Mammals	40.0	1.021	13	0.003–3.71
Mammals	46.0	1.059	33	0.003–700
Mammals	47.5	1.060	47	0.003–700
Canids	54.9	1.15	4	4.6–27.6
Dog (<i>Canis familiaris</i>)	52.8	1.07	1	2.65–57
Bats	96.4	1.07	5	0.005–0.173
$S(A)$, m²; Alveolar Surface Area				
Mammals	1.87	0.888	13	0.003–3.71
Mammals	3.34	0.949	33	0.003–700
Mammals	3.36	0.935	47	0.003–700
Canids	1.12	1.38	4	4.6–27.6
Dog (<i>C. familiaris</i>)	3.12	1.05	1	2.65–57
Bat	5.18	1.01	5	0.005–0.173
$S(c)$, m²; Pulmonary Capillary Surface Area				
Mammals	2.73	0.952	33	0.003–700
Mammals	2.72	0.941	47	0.003–700
Canids	1.14	1.25	4	4.6–27.6
Dog (<i>C. familiaris</i>)	2.53	1.05	1	2.65–57
$V(c)$, mL; Pulmonary Capillary Volume				
Mammals	3.20	1.000	33	0.003–700
Mammals	3.63	1.009	47	0.003–700
Canids	1.92	1.23	4	4.6–27.6
Dog (<i>C. familiaris</i>)	3.28	1.12	1	2.65–57
Bats	3.73	0.954	5	0.005–0.173
τ_{hr}, μm; Harmonic Mean Thickness of Alveolar Membrane				
Mammals	0.416	0.050	33	0.003–700
Mammals	0.413	0.053	47	0.003–700
Canids	0.476	0.000	4	4.6–27.6
Dog (<i>C. familiaris</i>)	0.39	0.80	1	2.65–57
Bats	0.249	0.021	5	0.005–0.173

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Notes: Allometry of pulmonary structural variables that contribute to gas conductance of the lung and morphometric estimates of pulmonary diffusing capacity (DL_0). Variables are related to body mass (M_b , in kg) by the function: $Y = a \cdot M_b^b$, where Y is the variable. a is the intercept of the log-transformed linear regression, and b is the slope of the log-transformed linear regression. N is the number of species. In some cases, regression equations were calculated from data given in the reference. Units have been converted for equivalence, where necessary.

TABLE 6.5
Total Tissue Volumes, Surface Areas, and Mean Tissue Thickness in the Alveolar Region
of Normal Mammalian Lungs^a

	Fischer-344 Rat (n = 4)	Sprague-Dawley Rat (n = 8)	Dog (n = 4)	Baboon (n = 5)	Human (n = 8)
Body weight, kg	0.29 ± 0.01 ^b	0.36 ± 0.01	16 ± 3	29 ± 3	74 ± 4
Lung volume, mL	8.6 ± 0.31 ^b	10.55 ± 0.37 ^b	1322 ± 64	2393 ± 100	4341 ± 284
Total volumes, cm ³ /both lungs					
Air	5.978 ± 0.197	7.216 ± 0.278 ^b	914 ± 52	1851 ± 24	3422 ± 223
Capillary lumen	0.649 ± 0.057 ^b	0.659 ± 0.055 ^b	92 ± 5 ^c	44 ± 17 ^c	169 ± 24
Tissue	0.428 ± 0.046 ^b	0.671 ± 0.041 ^b	78 ± 4 ^c	68 ± 9 ^c	314 ± 41
Type I epithelium	0.082 ± 0.006 ^b	0.144 ± 0.010 ^b	16.5 ± 1.9 ^c	14.4 ± 2.3 ^c	32.5 ± 3.9
Type II epithelium	0.037 ± 0.009 ^b	0.053 ± 0.009 ^b	5.6 ± 0.5 ^c	3.5 ± 0.8 ^c	32.1 ± 5.0
Cellular interstitium	0.068 ± 0.005 ^b	0.079 ± 0.015 ^b	12.9 ± 0.7 ^c	9.4 ± 1.6 ^c	54.0 ± 7.0
Noncellular interstitium	0.128 ± 0.016 ^b	0.214 ± 0.016 ^b	22.8 ± 0.7 ^c	24.6 ± 3.7 ^c	98.3 ± 12.4
Endothelium	0.094 ± 0.009 ^b	0.156 ± 0.007 ^b	17.6 ± 1.6	13.4 ± 2.6	42.6 ± 5.4
Macrophages	0.019 ± 0.007 ^b	0.025 ± 0.006 ^b	2.5 ± 1.1 ^c	2.4 ± 1.1 ^c	54.7 ± 15.7
Surface area, m ² both lungs ^d					
Alveolar epithelium					
Type I	0.391 ± 0.39 ^b	0.387 ± 0.025 ^b	51.0 ± 1.0 ^c	47.7 ± 7.7 ^c	89.0 ± 8.0
Type II	0.015 ± 0.005 ^b	0.015 ± 0.002 ^b	1.0 ± 0.2	1.9 ± 0.3	7.0 ± 1.0
Capillary endothelium	0.383 ± 0.39 ^b	0.452 ± 0.035 ^b	57.0 ± 2.0	38.6 ± 9.5	91.0 ± 9.0
Arithmetic mean, tissue thickness, μm					
Epithelium					
Type I	0.212 ± 0.008 ^c	0.384 ± 0.038 ^b	0.327 ± 0.043 ^{bc}	0.308 ± 0.021 ^{bc}	0.361 ± 0.024 ^b
Type II	2.758 ± 0.424 ^{bc}	3.653 ± 0.266 ^b	4.138 ± 0.340 ^{bc}	1.839 ± 0.141 ^c	5.019 ± 0.551 ^c
Interstitial	0.500 ± 0.028 ^b	0.693 ± 0.058 ^b	0.658 ± 0.033 ^b	0.847 ± 0.140 ^b	1.634 ± 0.164
Endothelium	0.246 ± 0.011 ^b	0.358 ± 0.031 ^b	0.308 ± 0.019 ^b	0.361 ± 0.038 ^{bc}	0.474 ± 0.052 ^c

Source: Pinkerton, K.E. et al., Architecture and cellular composition of the airblood barrier, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a All data are mean ± SEM. For comparisons between species all data connected by the same letter superscripts (b, c) are not statistically different from each other.

^d Type I surface area (SA) is the SA of basement membrane under type I cells; type II SA is the air surface of type II cells excluding the extra SA contributed by microvilli; endothelial SA is the luminal surface of the endothelial cells.

TABLE 6.6
Tracheobronchial and URT Liquid Lining Layer Thicknesses

Species	Location	Thickness ^a (μm)	Comments
Cat	Trachea	≤20, usually <10	Not uniform "in some areas not detected"
Guinea pig	Trachea	~10	"Constantly biophasic"
	Intrapulmonary	0.0	Liquid lining never extended beyond ciliary tips
Human	Bronchi (main stem)	8.3 ^b	Combination of airway and vascular fixation, lobes were surgical resections in nonsmokers
	Bronchi (segmental)	6.9 ^b	
	Bronchioles	1.8 ^b	
Rabbit	Bronchi (1–3 mm) ^c	5 or more ^b	
	Bronchioles (0.5–1.0 mm) ^c	1–4 ^b	Mucous blanket observed
	Bronchioles (<0.5 mm) ^c	0.3–0.5 ^b	
	Terminal bronchioles	<0.1 ^b	
Rat	Nose	≤15	Observed a continuous blanket
	Trachea large bronchi	5–10	"Distribution was focal"
	Trachea	3–15, generally 8–12 ^b	Mucous blanket observed

(continued)

TABLE 6.6 (continued)
Tracheobronchial and URT Liquid Lining Layer Thicknesses

Species	Location	Thickness ^a (μm)	Comments
	Lobar bronchi	Few tenths-8, generally 2–5 ^b	
	Trachea	6.1 ^b	
	Bronchi	3 ^b	Combination of airway and vascular fixation
	Bronchioles	2 ^b	
	Terminal bronchioles	0.0	No epiphase or mucus observed
	Trachea, major bronchi, peripheral airways		“Well-defined streams up to 500 μm wide.” “Mucus was transported as discrete particles” as small as 0.5 μm in diameter. A continuous mucous blanket was not observed.

Source: Miller, F.J. et al., Regional respiratory tract absorption of inhaled reactive gasses, 2nd edn., *Toxicology of the Lung*, Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.

^a Unless specified, values apply only to the epiphase above cilia tips.

^b Thickness of epiphase plus hypophase.

^c Diameter range of airways.

TABLE 6.7
Alveolar Region Tissue and Blood Compartment Dimensions of Normal Mammalian Lungs

	Fischer-344 Rat (n = 4)	Sprague-Dawley Rat (n = 8)	Dog (n = 4)	Baboon (n = 5)	Human (n = 8)
Body weight (kg)	0.29 ± 0.01 ^a	0.36 ± 0.01	16 ± 3	29 ± 3	79 ± 4
Lung volume (mL)	8.6 ± 0.31	10.55 ± 0.37	1,322 ± 64	2,393 ± 100	4,341 ± 284
Total volumes (cm ³ both lungs)					
Air	5.978 ± 0.197	7.216 ± 0.278	914 ± 52	1,851 ± 24	3,422 ± 223
Capillary lumen	0.649 ± 0.057	0.659 ± 0.055	92 ± 5	44 ± 17	169 ± 24
Tissue	0.428 ± 0.046	0.671 ± 0.041	78 ± 4	68 ± 9	314 ± 41
Type I epithelium	0.082 ± 0.006	0.144 ± 0.010	16.5 ± 1.9	14.4 ± 2.3	32.5 ± 3.9
Type II epithelium	0.037 ± 0.009	0.053 ± 0.009	5.6 ± 0.5	3.5 ± 0.8	32.1 ± 5.0
Cellular interstitium	0.068 ± 0.005	0.079 ± 0.015	12.9 ± 0.7	9.4 ± 1.6	54.0 ± 7.0
Noncellular interstitium	0.128 ± 0.016	0.214 ± 0.016	22.8 ± 0.7	24.6 ± 3.7	98.3 ± 12.4
Endothelium	0.094 ± 0.009	0.156 ± 0.007	17.6 ± 1.6	134 ± 2.6	42.6 ± 5.4
Macrophages	0.019 ± 0.007	0.025 ± 0.006	2.5 ± 1.1	2.4 ± 1.1	54.7 ± 15.7
Surface area (m ² /both lungs)					
Alveolar epithelium					
Type I	0.391 ± 0.039	0.387 ± 0.025	51.0 ± 1.0	47.7 ± 7.7	89.0 ± 8.0
Type II	0.015 ± 0.005	0.015 ± 0.002	1.0 ± 0.2	1.9 ± 0.3	7.0 ± 1.0
Capillary endothelium	0.383 ± 0.039	0.452 ± 0.035	57.0 ± 2.0	38.6 ± 9.5	91.0 ± 9.0
Tissue component of diffusion capacity (mL O ₂ min ⁻¹ mm Hg ⁻¹)	3.43 ± 0.17	3.55 ± 0.33	399 ± 12	23.1 ± 5.2	436 ± 53.6
Tissue thickness (μm)					
Harmonic mean (air/plasma)	0.379 ± 0.030	0.405 ± 0.017	0.450 ± 0.007	0.674 ± 0.055	0.745 ± 0.059
Arithmetic mean					
Epithelium					
Type I	0.212 ± 0.008	0.384 ± 0.038	0.327 ± 0.043	0.308 ± 0.021	0.361 ± 0.024
Type II	2.578 ± 0.424	3.653 ± 0.266	4.138 ± 0.340	1.839 ± 0.141	5.019 ± 0.551
Interstitial	0.500 ± 0.028	0.693 ± 0.058	0.658 ± 0.033	0.847 ± 0.140	1.634 ± 0.164
Endothelium	0.246 ± 0.011	0.358 ± 0.031	0.308 ± 0.019	0.361 ± 0.038	0.474 ± 0.052

Sources: Crapo, J.D. et al., *Am. Rev. Respir. Dis.*, 128, 542, 1983; Miller, F.J. et al., Regional respiratory tract absorption of inhaled reactive gasses, in *Toxicology of the Lung*, Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.

^a All data are mean ± SEM.

TABLE 6.8
Anatomical Data: Trachea of Various Species

Species	Length (cm)	Internal Diameter (mm)	Goblet Cells per cm	Glands
Dog	10	15	350	+++
Baboon	3.5	5	600	+++
Pig	13	10	150	+++
Macaca	6	7	300	++
Mouse	2	2	—	±
Rabbit	6.5	10	150	—
Guinea pig	3.3	4	600	—
Hamster	1.5	2	6	—
Sheep	24	26	300	+++
Cat (SPF)	7	7	600	++++
Squirrel monkey	2.5	3	150	+
Rat	3.2	3	8	±
Man	11.0	20	200 ^a	+++ ^a

Source: Phalen, R.F., *Inhalation Studies: Foundations and Techniques*, CRC Press, Boca Raton, FL, 1984. With permission.

^a Varies considerably.

TABLE 6.9
Dimensions of the Cross Sections of the Nasal Cast of the Rat

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
1	0	8.3	1.4
2	1	5.6	1.3
3	2	11.9	2.0
4	3	21.7	3.5
5	4	23.4	3.8
6	5	27.3	4.5
7	5.5	35.0	7.6
8	7	38.3	9.9
9	8	44.0	9.3
10	10	73.9	13.2
11	12	63.5	10.3
12	14	54.4	10.3
13	16	48.3	8.1
14	17	67.6	16.7
14 ^a	17	57.8	13.0
15	18	58.4	21.7
16	19	104.1	25.8
16 ^a	19	47.5	14.0
17	20	97.8	17.0
17 ^a	20	9.0	5.0
18	21	44.5	12.5
18 ^a	21	9.0	5.0
19	22	30.9	7.7
19 ^a	22	8.5	4.2
20	23	17.4	5.9
20 ^a	23	8.9	4.9
21	25	7.5	3.6
22	32	7.9	3.7
23	35	8.6	2.8
24	37	7.7	2.2
25	40	8.4	3.4

TABLE 6.9 (continued)
Dimensions of the Cross Sections of the Nasal Cast of the Rat

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
26	41	8.7	3.4
27	42	23.7	15.8
28	43	24.2	14.9
29	44	9.2	3.2
30	44.5	12.8	3.2
31	45	7.6	1.8
32	45.5	7.4	1.9
33	46	7.8	3.1
34	47	6.5	3.0
35	52	7.1	3.8

Source: Reproduced from Schreider, J.P., *Toxicology of the Nasal Passages*, Hemisphere Publishing, Taylor & Francis, Washington, DC, p. 10, 1986. With permission. All rights reserved.

^a Main airway alone. Does not include separate pockets.

TABLE 6.10
Dimensions of the Cross Sections of the Nasal Cast of the Guinea Pig

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
1	0	18.0	5.0
2	1	14.0	4.4
3	2	15.3	4.9
4	2.5	12.4	3.6
5	3	13.9	4.2
6	3.5	18.5	5.4
7	4	32.9	13.0
8	5.0	19.8	13.4
9	8	66.4	20.4
10	11	88.0	21.5
11	16	91.5	28.5
12	18	91.2	31.1
13	20	82.0	36.2
14	22	62.7	30.1
15	24	75.1	38.1
16	26	109.4	44.4
16 ^a	26	94.3	39.2
17	28	153.5	58.1
17 ^a	28	14.9	11.2
18	31	184.6	73.4
18 ^a	31	14.5	10.8
19	34	14.2	13.3
19 ^a	34	8.2	11.7
20	39	13.9	13.7
21	45	13.2	31.2
22	50	12.0	8.1
23	55	8.0	4.1
24	56	18.9	8.8

(continued)

TABLE 6.10 (continued)
Dimensions of the Cross Sections of the Nasal Cast
of the Guinea Pig

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
25	57	20.7	12.7
26	59	8.9	2.8
27	62	9.1	5.1
28	70	8.6	5.7

Source: Reproduced from Schreider, J.P., *Toxicology of the Nasal Passages*, Hemisphere Publishing, Taylor & Francis, Washington, DC, p. 11, 1986. With permission. All rights reserved.

^a Main airway alone. Does not include side pockets.

TABLE 6.11
Dimensions of the Cross Sections of the Nasal Cast
of the Beagle

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
1	0	44.0	33.3
2	5	50.3	42.3
3	10	72.5	44.8
4	15	66.5	32.0
5	20	63.8	37.6
6	25	70.1	49.6
7	30	106.3	75.9
8	35	143.4	95.5
9	40	294.8	155.0
10	43	503.0	205.8
11	50	674.3	228.4
11 ^a	50	665.6	227.3
12	55	470.6	267.3
13	60	333.9	312.5
14	65	318.8	240.9
14 ^a	65	64.3	91.2
15	70	424.1	277.5
15 ^a	70	55.1	68.0
16	75	266.8	289.5
16 ^a	75	109.1	143.8
17	80	200.9	272.5
17 ^a	60	54.5	113.9
18	90	121.5	138.3
18 ^a	90	52.6	105.3
19	100	44.4	95.2
19 ^a	100	35.6	92.6
20	120	39.3	88.8
21	150	64.0	139.1
22	160	184.5	1022.5
23	170	131.0	871.3
24	180	210.0	1055.4
25	185	250.9	933.6
25 ^a	185	53.1	89.1
26	190	50.8	112.6

TABLE 6.11 (continued)
Dimensions of the Cross Sections of the Nasal Cast
of the Beagle

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
27	195	50.6	175.4
28	205	57.5	251.3
29	275	57.0	237.1

Source: Reproduced from Schreider, J.P., *Toxicology of the Nasal Passages*, Hemisphere Publishing, Taylor & Francis, Washington, DC, p. 11, 1986. With permission. All rights reserved.

^a Main airway alone. Does not include separate pockets.

TABLE 6.12
Dimensions of the Cross Sections of the Nasal Cast
of the Rhesus Monkey

Section No.	Distance from Anterior End of Nose (mm)	Perimeter (mm)	Area (mm ²)
1	0	45.8	55.6
2	3	43.3	51.0
3	8	62.3	61.0
4	13	73.8	86.2
5	18	95.6	105.6
6	23	119.3	161.1
7	25	121.0	155.0
8	28	169.1	180.8
9	33	192.9	165.6
10	38	211.8	165.3
11	40	171.8	164.1
12	43	143.4	161.7
13	48	116.1	152.6
14	53	58.5	91.5
15	58	45.0	53.2
16	63	37.9	38.0
17	68	24.0	18.8
18	73	29.0	27.3
19	78	37.9	66.0
20	80	66.5	224.9
21	83	73.6	253.1
22	88	90.5	263.0
22 ^a	88	75.4	256.4
23	90	118.8	257.3
24	93	34.1	43.8
25	95	27.6	15.8
26	98	29.5	18.2
27	103	35.0	72.0
28	105	33.3	75.4
29	110	27.9	56.1

Source: Reproduced from Schreider, J.P., *Toxicology of the Nasal Passages*, Hemisphere Publishing, Taylor & Francis, Washington, DC, p. 13, 1986. With permission. All rights reserved.

^a Main airway alone. Does not include separate pockets.

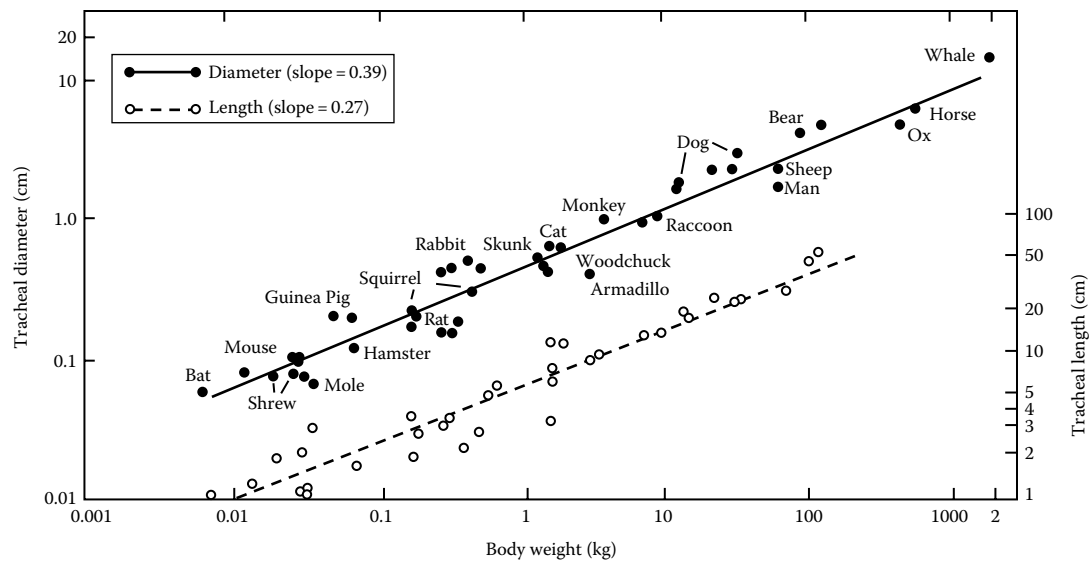


FIGURE 6.1 Relationship between body mass and the logarithms of tracheal diameter and length for a variety of species. (From Tenney, S.M. and Bartlett, D., *Respir. Physiol.*, 3, 130, 1967. With permission.)

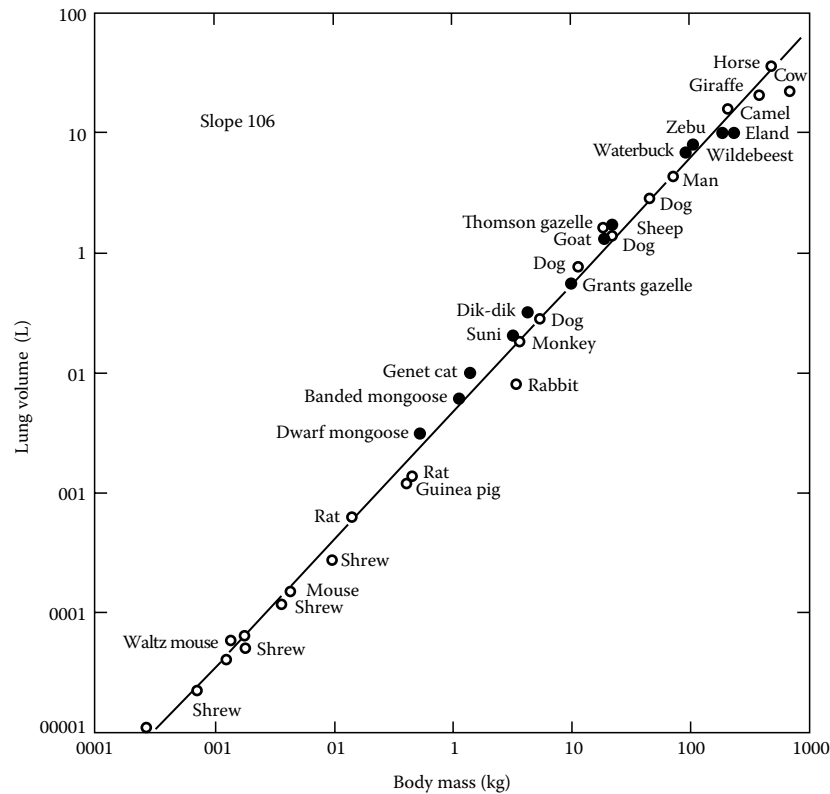


FIGURE 6.2 Allometric plot of mean lung volume to mean body mass for mammalian species. Closed circles are African species and open circles are other species. (From Gehr, P. et al., *J. Respir. Physiol.*, 44, 61, 1981. With permission.)

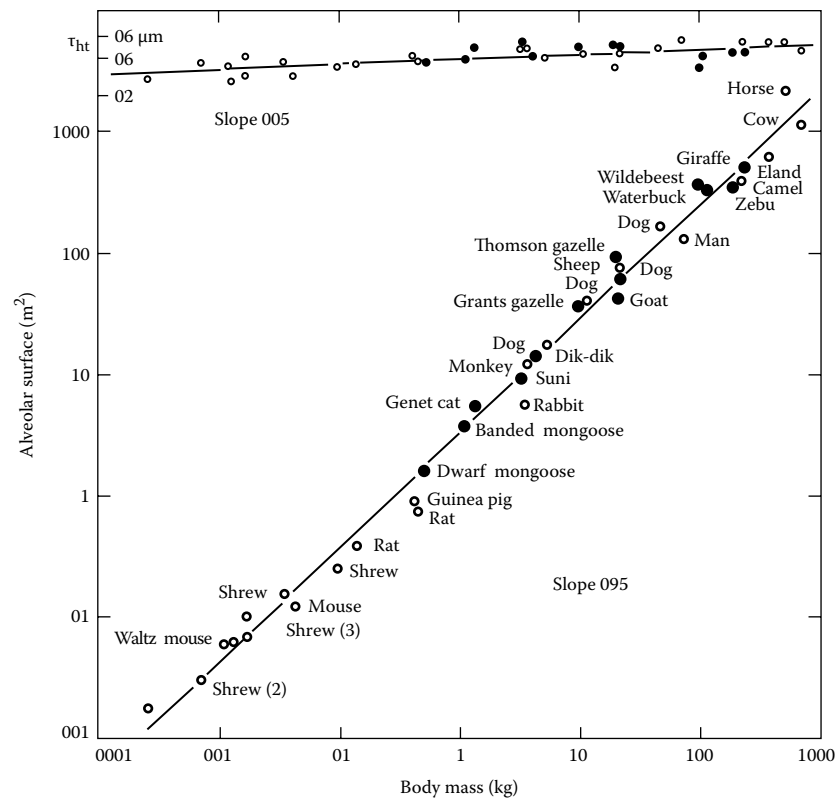


FIGURE 6.3 Allometric plot of alveolar surface area and harmonic mean tissue thickness (T_{ht}) to body mass for mammalian species. (From Gehr, P. et al., *J. Respir. Physiol.*, 44, 61, 1981. With permission.)

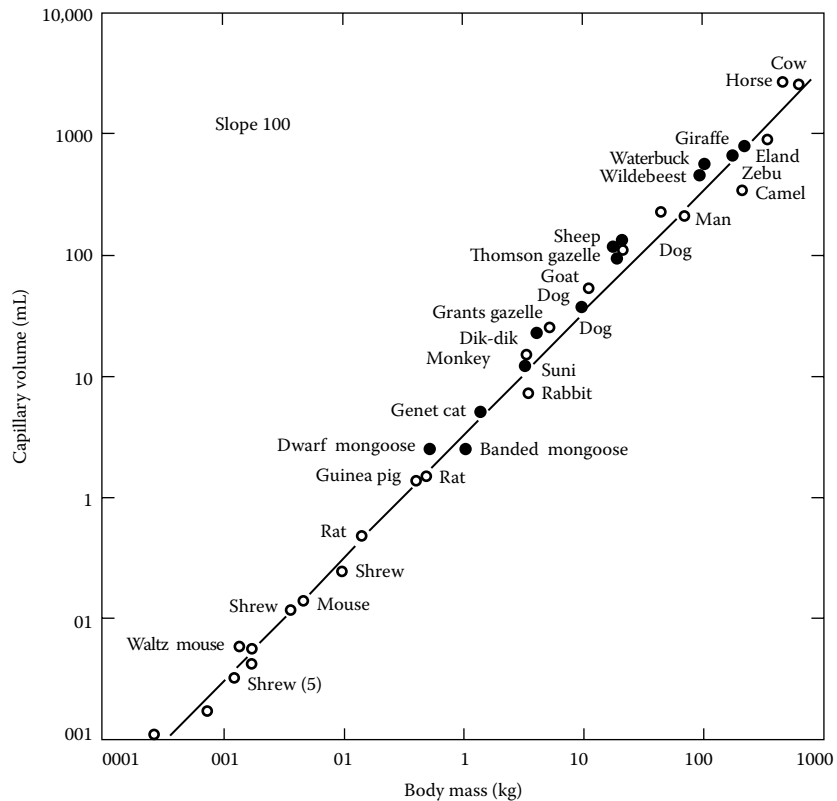


FIGURE 6.4 Allometric plot of capillary volume to body mass for mammalian species. (From Gehr, P. et al., *J. Respir. Physiol.*, 44, 61, 1981. With permission.)

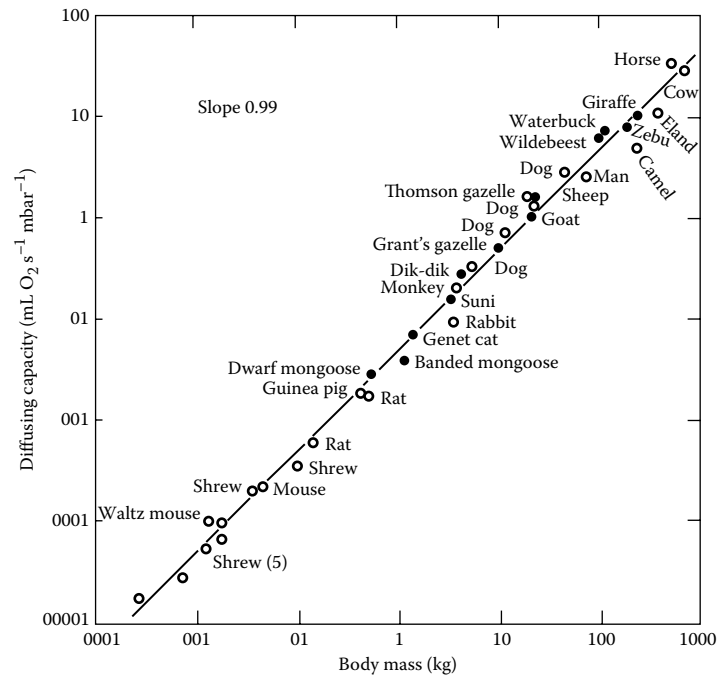


FIGURE 6.5 Allometric plot of pulmonary diffusing capacity to body mass for mammalian species. (From Gehr, P. et al., *J. Respir. Physiol.*, 44, 61, 1981. With permission.)

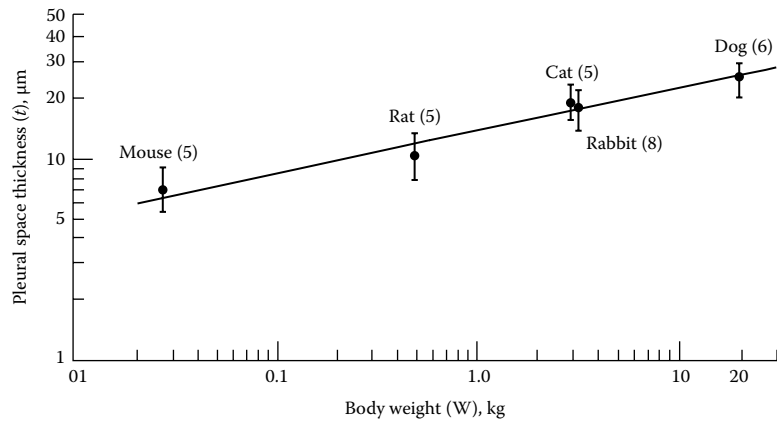


FIGURE 6.6 Pleural space thickness (*t*) vs. body weight (*W*) measured using light microscopy in five mammalian species. The power curve fit to the data was $t = 13.1 W^{0.2}$. (From Li-Fook, et al., *J. Appl. Physiol.*, 59, 603, 1985. With permission.)

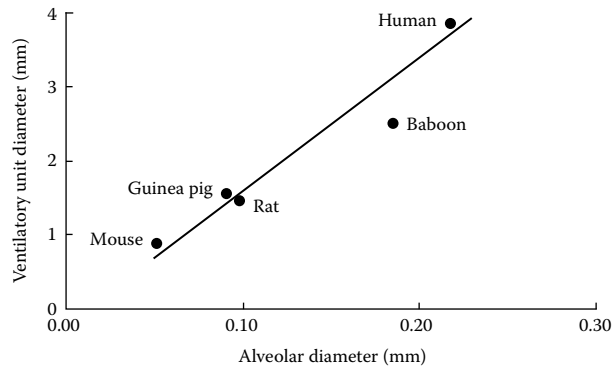


FIGURE 6.7 Mean alveolar diameter vs. mean ventilatory unit diameter. The alveolar diameters and corresponding ventilatory unit diameters from the lungs of different species are shown. The lungs were preserved by vascular perfusion fixation at a lung volume near functional residual capacity. A remarkable similarity in lung structure across species is demonstrated by the fact that the ratio of ventilatory unit diameter to alveolar diameter is constant over the large range of lung sizes examined. The ventilatory unit averaged 17.5 alveolar diameters in size. (From Pinkerton, K.E. et al., *J. Appl. Physiol.*, 1992. With permission.)

CELLS IN THE RESPIRATORY TRACT

(TABLES 6.13 THROUGH 6.25; FIGURE 6.8)

TABLE 6.13

Comparison of Abundance and Percentage of Cell Types in Tracheas of Seven Mammalian Species

Species	N	Total Nuclei	Percentage						
			Basal Cells	Ciliated Cells	Clara Cells	Mucous Goblet Cells	Serous Cells	Other Cells	Unidentified
Sheep	5	414.3 ^a ± 33.2	28.5	30.6	0	5.1	0	35.9	0
Bonnet monkey	3	266.0 ± 12.0	31	41	0	8	0	16	4
Rhesus monkey	5	181.4 ± 50.7	42.0	32.9	0	16.8	0	4.3	4.0
Cat	3	273.0 ± 15.0	37.3	36.1	0	20.2	0	5.4	1.1
Rabbit	3	210.9 ± 29.7	28.2	43.00	17.6	1.3	0	0	9.4
Rat	3	147.9 ± 3.1	13.4	40.6	0	0.5	39.2	0	6.2
Hamster	3	151.4 ± 11.2	5.6	47.5	41.4	0	0	0	5.3

Sources: Plopper, C.G. et al., *Exp. Lung Res.*, 5, 281, 1983; St. George, J.A. et al., Cell populations and structural function relationships of cells in the airways, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.

^a Mean ± SD, number of nuclei per millimeter.

TABLE 6.14

Density of Cells in the Bronchiolar Epithelium of Adults

Species	Cell Density/mm ^{2a}	
	All Cell Types	Nonciliated Cells
Cat	19,532 ± 383	19,532 ± 383
Rat	18,813 ± 2,722	14,030 ± 3,373
Rabbit	15,073 ± 706	9,261 ± 434
Hamster	14,238 ± 2,794	8,489 ± 2,407
Mouse	9,759 ± 1,700	8,732 ± 2,200
Bonnet monkey	9,565 ± 304	8,800 ± 280

Source: St. George, J.A. et al., Cell populations and structural function relationships of cells in the airways, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.

^a Mean ± SD.

TABLE 6.15

Height (μm) of the Airway Epithelium in Adult Mammals from Various Reports

		Trachea		Bronchi					
		N	(Generation 0)	N	(Generation 1–2)	N	(Generation 4–6)	N	(Generation 7–11)
Primates									
Human	3	50–100; 43 ± 2	5	50–; 39–	—	—	—	—	
Bonnet monkey (<i>M. radiata</i>)	3	20 ± 7	3	21 ± 6	—	—	—	—	
Rhesus monkey (<i>M. mulatta</i>)	5	28 ± 5	5	19 ± 1	5	17 ± 1	5	13 ± 1	
Carnivores									
Dog	5	26 ± 4	—	20–50	—	—	—	—	
Cat	5	20–24; 24±	—	—	—	—	—	—	
Ferret	4	—	—	—	—	—	—	—	

TABLE 6.15 (continued)

Height (μm) of the Airway Epithelium in Adult Mammals from Various Reports

	Trachea		Bronchi					
	N	(Generation 0)	N	(Generation 1–2)	N	(Generation 4–6)	N	(Generation 7–11)
Perissodactyla								
Horse	3	48 \pm 3	—	—	—	—	—	—
Artiodactyla								
Cow	5	60 \pm 3	—	—	—	—	—	—
Sheep	5	41–57; 59 \pm 7 ^a	6	40 \pm 3	8	32 \pm 4 ^a	6	30 \pm 5 ^a
Pig	5	46 \pm 5	3	35–50	—	—	—	—
Rodentia								
Guinea pig	5	30 \pm 3	—	15–30	—	—	—	—
Hamster	3	20 \pm 0; 8–14	—	151 \pm 11	5	20–	—	—
Rat	3	24 \pm 1	20	13–	6	13–	—	—
Mouse	6	11–14	—	—	3	8–16	—	—
Lagomorpha								
Rabbit	—	21 \pm 1	4	22 \pm 3	6	9 \pm 2	5	9 \pm 1

Source: Mariassy, A.T., Epithelial cells of the trachea and bronchi, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Insufflation fixed, epon-embedded trachea (\pm SD).

TABLE 6.16

Population Densities (% Cells) of the Tracheal Surface Epithelium in Adult Mammals and Juvenile Birds

	N	Epithelial Cells								
		Cells/mm Basal Lamina	Basal	Ciliated	Mucous (Goblet)	Serous	Other ^a	Clara	Brush	Unidentified
Primates										
Human	3	303 \pm 20	33	49	9	0	9	0	—	—
Bonnet monkey (<i>Macaca radiata</i>)	3	266 \pm 12	31	41	0	0	16 SMGC ^b	0	<1	4
Rhesus monkey (<i>Macaca mulatta</i>)	5	181 \pm 51	42	33	17	0	4 SMGC	0	<1	4
Carnivores										
Dog	5	—	—	—	—	—	—	—	—	—
Cat	5	273 \pm 15	37	36	20	0	5	0	<1	1
Ferret	4	183–	25	54	22	0	—	0	Rare	—
Perissodactyla										
Horse	3	307 \pm 23	31	46	5	0	18	0	—	—
Artiodactyla										
Cow	5	323 \pm 24	31	42	4	0	23	0	<1	—
Sheep	5	414 \pm 33	29	31	5	0	36 M3 ^c	0	<1	0
Pig	5	303 \pm 17	31	43	3	0	23	0	<1	—
Rodentia										
Guinea pig	5	307 \pm 5	34	32	5	0	29	0	<0	—
Hamster	3	151 \pm 11	6	48	0	0	0	41	<1	5
Rat	3	148 \pm 3	13	41	1	39	0	0	1	6
Rat	5	168 \pm 12	21	32	2	42	1	0	>1	1
Rat	—	142–	27	33	<1	27	13	0	—	—
Mouse	6	215–	10	39	<1	0	1	49	<1	—
Lagomorpha										
Rabbit	—	211 \pm 30	28	43	1	0	0	18	Rare	9
Avia										
Goose	—	213–	33	50	12	<0.01	0	0	0	5

Source: Mariassy, A.T., Epithelial cells of the trachea and bronchi, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: \pm SD

^a Nonciliated cells, mostly eccrine.

^b Small mucous granule cell.

^c Mucous cell.

Population Densities (% Cells) of the Bronchial Surface Epithelium in Adult Mammals (Mainstem, Primary Bronchi)

^c Mucous cell.

Population Densities (% Cells) of the Bronchial Surface Epithelium in Adult Mammals (Lobar Bronchi, Generations 2–6)

[illegible]

TABLE 6.18 (continued)
Population Densities (% Cells) of the Bronchial Surface Epithelium in Adult Mammals
(Lobar Bronchi, Generations 2–6)

	N	Cells/mm Basal Lamina	Epithelial Cells							
			Basal	Ciliated	Mucous (Goblet)	Serous	Other ^a	Clara	Brush	Unidentified
Rodentia										
Guinea pig	—	—	—	—	—	—	—	—	—	—
Hamster	5	179–	18	35	27	0	12	7	<1	1
Rat	6	116–	14	53	<1	20	12	0	Infrequent	
Mouse	3	199–	1	36	0	0	2	61	<1	<1
Lagomorpha										
Rabbit	6	114 ± 12	2	49	0	0	7	41	<1	—

Source: Mariassy, A.T., Epithelial cells of the trachea and bronchi, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: ±SD.

^a Nonciliated, mostly eccrine.

^b Small mucous granule cell.

^c Mucous cell.

TABLE 6.19
Population Densities (% Cells) of the Bronchial Surface Epithelium in Adult Mammals
(Segmental Bronchi, Generations 7–11)

	N	Cells/mm Basal Lamina	Epithelial Cells							
			Basal	Ciliated	Mucous (Goblet)	Serous	Other ^a	Clara	Brush	Unidentified
Primates										
Human	—	—	—	—	—	—	—	—	—	—
Bonnet monkey (<i>Macaca radiata</i>)	—	—	—	—	—	—	—	—	—	—
Rhesus monkey (<i>Macaca mulatta</i>)	5	158 ± 15	29	49	14	0	3 SMGC ^b	0	—	2
Carnivores										
Dog	—	—	—	—	—	—	—	—	—	—
Cat	—	—	—	—	—	—	—	—	—	—
Ferret	—	—	—	—	—	—	—	—	—	—
Perissodactyla										
Horse	—	—	—	—	—	—	—	—	—	—
Artiodactyla										
Cow	—	—	—	—	—	—	—	—	—	—
Sheep	8	223 ± 17	18	43	8	0	31 M3 ^c	0	<1	0
Pig	—	—	—	—	—	—	—	—	—	—
Rodentia										
Guinea pig	—	—	—	—	—	—	—	—	—	—
Hamster	—	—	—	—	—	—	—	—	—	—
Rat	—	—	—	—	—	—	—	—	—	—
Mouse	—	—	—	—	—	—	—	—	—	—
Lagomorpha										
Rabbit	5	147 ± 18	0	49	0	0	4	47	—	0

Source: Mariassy, A.T., Epithelial cells of the trachea and bronchi, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: ±SD.

^a Nonciliated, mostly eccrine.

^b Small mucous granule cell.

^c Mucous cell.

TABLE 6.20

Comparison of Species Differences in Microenvironment of Bronchiolar Cells: Centriacinar Organization and Tracheobronchial Distribution

Species	Transitional or Respiratory Bronchiole		Nonciliated Bronchiolar Cells Found in	
	Extensive	Minimal	Trachea	Lobar Bronchus
Mouse		+	>50%	>60%
Hamster		+	>40%	>15%
Rat		+	0	0
Guinea pig		+	0	0
Rabbit		+	>15%	>25%
Dog	+		0	0
Cat	+		0	0
Ferret	+		0	0
Macaque	+		0	0
Monkey				
Sheep	+	+	0	0
Pig		+	0	0
Horse		+	0	0
Cow		+	0	0
Human			0	0

Source: From Plopper, C.G. and Hyde, D.M., Epithelial cells of bronchioles, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

TABLE 6.22

Comparison of Numerical Density of Bronchiolar Epithelium and Density and Percentage of Clara Cells in Bronchiolar Epithelial Population of Adults

Species	Bronchiolar Epithelium Density ^a (No./mm ²)	Clara Cells	
		Density ^a (No./mm ²)	% of Population
Rat	17,070 ± 791	4,336 ± 201	25.4
Rabbit	15,073 ± 706	9,261 ± 434	61.4
Cat	19,532 ± 383	19,532 ± 383	100
Bonnet monkey	9,565 ± 304	8,800 ± 280	92

Source: Plopper, C.G. and Hyde, D.M., Epithelial cells of bronchioles, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Mean ± SD.

TABLE 6.21

Comparison of Species Differences in Cellular Composition of Centriacinar Bronchiolar Epithelium

Species	Cell Types in Terminal Bronchiole				Cell Types in Respiratory Bronchiole			
	Clara	Ciliated	Goblet	Basal	Clara	Ciliated	Goblet	Basal
Mouse	>50%	<50%	—	—	N/A	N/A	N/A	N/A
Hamster	>50%	<50%	—	—	N/A	N/A	N/A	N/A
Rat	>50%	<50%	—	—	N/A	N/A	N/A	N/A
Guinea pig	>50%	<50%	—	—	N/A	N/A	N/A	N/A
Rabbit	>50%	<50%	—	—	N/A	N/A	N/A	N/A
Dog	>95%	<5%	—	—	>95%	<5%	—	—
Cat	>95%	<5%	—	—	>95%	<5%	—	—
Macaque monkey	—	~50%	~20%	~10%	>90%	<10%	+	+
Sheep	>60%	<40%	—	—	N/A	N/A	N/A	N/A
Pig	+	+	—	—	N/A	N/A	N/A	N/A
Horse	>50%	>50%	—	—	N/A	N/A	N/A	N/A
Cow	>50%	<50%	—	—	N/A	N/A	N/A	N/A
Human	—	+	+	+	+	+	+	+

Source: Plopper, C.G. and Hyde, D.M., Epithelial cells of bronchioles, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: N/A, not applicable; —, not present; +, present in variable amounts.

TABLE 6.23

Comparison of Relative Proportions (Percentages)^a of Cellular Components in Clara Cells

Species	Nucleus	Agranular Endoplasmic Reticulum	Secretory Granules	Cytoplasmic Glycogen	Mitochondria	Large Mitochondria	Lateral Cytoplasmic Extensions
Mouse	21.8 ± 6.5	54.8 ± 7.5	+	0	34.7 ± 6.4	+	+
Hamster	25.2 ± 6.1	79.3 ± 7.6	+	0	10.7 ± 4.4	—	+
Rat	28.5 ± 10.4	66.2 ± 9.4	+	0.1 ± 0.4	16.3 ± 6.0	+	+
Guinea pig	28.6 ± 8.9	58.3 ± 9.0	+	0 ± 8.5	25.1	+	+
Rabbit	23.8 ± 8.8	61.6 ± 5.4	+	7.0 ± 5.4	19.1 ± 7.6	+	+
Dog	23.4 ± 11.4	24.7 ± 8.6	+	57.1 ± 13.7	8.0 ± 6.7	—	+
Cat	26.7 ± 9.2	10.7 ± 6.7	—	61.3 ± 10.1	19.5 ± 9.6	+	+
Macaque monkey	28.6 ± 4.4	5.2 ± 3.3	+	0	14.1 ± 2.8	—	+
Sheep	26.6 ± 10.3	64.6 ± 18.3	+	6.8 ± 12.1	13.8 ± 8.8	—	+
Pig	?	?	+	?	?	+	+
Horse	8.6 ± 4.1	70.6 ± 4.5	+	0	10.6 ± 4.4	—	+
Cow	27.7 ± 10.1	21.9 ± 10.1	+	62.3 ± 11.5	12.0 ± 7.5	—	+
Human	41.9 ± 10.0	3.1 ± 3.5	+	4.6 ± 5.8	15.3 ± 6.6	—	+

Source: Plopper, C.G. and Hyde, D.M., Epithelial cells of bronchioles, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a As percent of cytoplasmic volume; mean ± SD; +, as present; —, not present.

TABLE 6.24

Characteristics of Cells from the Alveolar Region of Normal Mammalian Lungs^a

	Fischer-344 Rat	Sprague-Dawley Rat	Dog	Baboon	Human
Total number of cells/lung, 10 ⁹	0.67 ± 0.02 ^b	0.89 ± 0.04 ^b	114 ± 13 ^c	99 ± 9 ^c	230 ± 25
Total lung cells, %					
Alveolar type I	8.1 ± 0.3 ^b	8.9 ± 0.9 ^b	12.5 ± 1.7 ^c	11.8 ± 0.6 ^c	8.3 ± 0.6 ^b
Alveolar type II	12.1 ± 0.7 ^b	14.2 ± 0.7 ^{bc}	11.8 ± 0.6 ^b	7.7 ± 1.0	15.9 ± 0.8 ^c
Endothelial	51.1 ± 1.7 ^b	42.2 ± 1.1 ^b	45.7 ± 0.8 ^b	36.3 ± 2.4	30.2 ± 2.4
Interstitial	24.4 ± 0.7 ^b	27.7 ± 1.8 ^b	26.6 ± 0.7 ^b	41.8 ± 2.7	36.1 ± 1.0
Macrophage	4.3 ± 1.0 ^b	3.0 ± 0.3 ^b	3.4 ± 0.6 ^b	2.3 ± 0.7 ^b	9.4 ± 2.2
Alveolar surface covered, %					
Alveolar type I	96.4 ± 0.5 ^b	96.2 ± 0.5 ^b	97.3 ± 0.4 ^b	96.0 ± 0.6 ^b	92.9 ± 1.0
Alveolar type II	3.6 ± 0.3 ^b	3.8 ± 0.5 ^b	2.7 ± 1.0 ^b	4.0 ± 0.6 ^b	7.1 ± 1.0 ^b
Average cell volume, μm ³					
Alveolar type I	1530 ± 121 ^b	2042 ± 374 ^b	1196 ± 88 ^b	1224 ± 136 ^b	1764 ± 155
Alveolar type II	455 ± 108 ^b	443 ± 80 ^b	428 ± 37 ^b	539 ± 184 ^b	889 ± 101
Endothelial	275 ± 25 ^b	387 ± 30 ^b	343 ± 19 ^b	365 ± 61 ^b	632 ± 64
Interstitial	427 ± 55 ^b	331 ± 67 ^{bc}	440 ± 47 ^b	227 ± 30 ^c	637 ± 26
Macrophage	639 ± 131 ^b	1058 ± 257 ^b	654 ± 116 ^b	1059 ± 287 ^b	2492 ± 167
Average cell surface area, μm ²					
Alveolar type I	7287 ± 755 ^c	5320 ± 694 ^{bc}	3794 ± 487 ^b	4004 ± 383 ^b	5098 ± 659 ^b
Alveolar type II	185 ± 56 ^{bc}	123 ± 20 ^b	107 ± 15 ^b	285 ± 85 ^c	183 ± 14 ^{bc}
Endothelial	1121 ± 95 ^b	1105 ± 72 ^b	1137 ± 127 ^b	1040 ± 209 ^b	1353 ± 67 ^b

Source: Pinkerton, K.E. et al., Architecture and cellular composition of the airblood barrier, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: Modified from Crapo, J.D. et al. (1983)¹⁰

^a All data are mean ± SEM. For comparisons between species

^{bc} Indicate those values that are not different from other values having the same letter superscript.

TABLE 6.25
Summary of Experimentally Determined Turnover Times for Selected Cells of Respiratory Tracts of Rats and Mice

Tissue	Turnover Time (Days)	
	Rat	Mouse
Tracheal epithelium	7–48	2–20
Bronchial epithelium	8–27	2–21
Bronchiolar epithelium	—	10–59
Alveolar epithelium	29	28–35
Alveolar macrophage	8–35	6–21

Source: Snipes, M.B., *Crit. Rev. Toxicol.*, 20, 174, 1989. With permission.

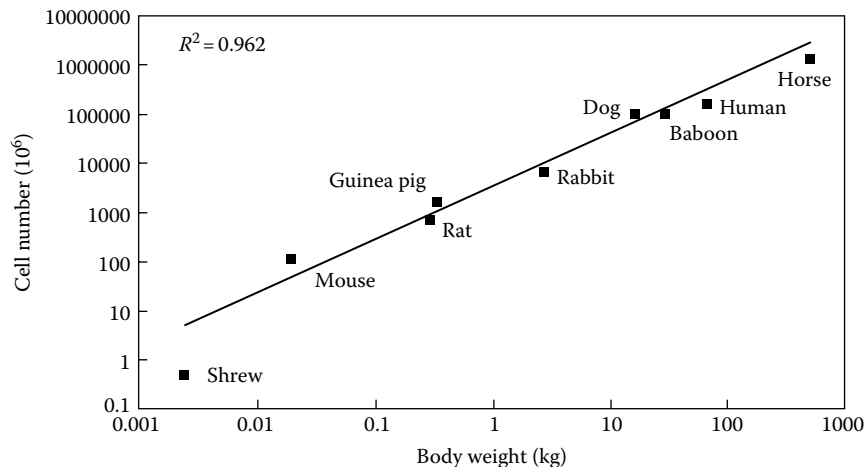


FIGURE 6.8 Allometric relationship for alveolar cells. On a log/log scale, the total number of alveolar cells within a species increases proportionally with body weight. For total number of lung cells, the slope is near 1 (0.95 ± 0.05) and statistically significant ($P < .0001$). A similar relationship holds for epithelial type I, epithelial type II, and interstitial and endothelial cells. A larger lung is therefore composed of more cells, not necessarily larger cells. (From Stone, K.C. et al., *Am. J. Respir. Cell. Mol. Biol.*, 6, 235, 1992. With permission.)

PULMONARY FUNCTION
(TABLES 6.26 THROUGH 6.39)

TABLE 6.26
Allometry of Pulmonary Diffusing Capacity

Taxon	Method	Intercept	Slope	N	M_b Range
DL _{co} mL [STPD] CO · min ⁻¹ · Torr ⁻¹ ; Pulmonary Diffusing Capacity for CO (Physiologic)					
Mammals	—	0.22	1.14	—	—
Mammals	—	0.614	0.985	—	0.025–500
Canids	sb	0.592	1.275	4	4.6–27.6
Laboratory mammals	sb	0.4680	0.74	4	0.040–3.50
Dog (<i>Canis familiaris</i>)	sb	1.67	0.79	1	7.3–63
Rabbit (<i>Oryctolagus cuniculus</i>)	sb	0.4074	0.92	1	1.30–3.50
Guinea pig (<i>Cavia porcellus</i>)	sb	0.4571	0.74	1	0.250–1.000
Rat (<i>Rattus norvegicus</i>)	sb	0.4467	0.75	1	0.050–0.400

TABLE 6.26 (continued)
Allometry of Pulmonary Diffusing Capacity

Taxon	Method	Intercept	Slope	N	M_b Range
DL _{co} mL [STPD] CO · min ⁻¹ · Torr ⁻¹ ; Pulmonary Diffusing Capacity for CO (Physiologic)					
Hamster (<i>Mesocricetus auratus</i>)	sb	0.7943	0.91	1	0.040–0.120
Mouse (<i>Mus musculus</i>)	rb	0.00106	0.971	1	0.020–0.039
Mammals		0.16	1.18	—	—
Mammals		3.92	0.991	33	0.003–700
Mammals		6.49	0.962	47	0.003–700
Canids		1.92	1.25	4	4.6–27.6
Dog (<i>C. familiaris</i>)		6.56	1.00	1	2.65–57
Bats		6.25	0.932	5	0.005–0.173

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Notes: Allometry of pulmonary diffusing capacity for carbon monoxide (DL_{co}, physiologic) and oxygen (DL_{O₂}, morphometric); both are in mL [STPD] · min⁻¹ · Torr⁻¹. DL is related to body mass (M_b in kg) by the function: $DL = a \cdot M_b^b$, where a is the intercept of the log-transformed linear regression, and b is the slope of the log-transformed linear regression. Method indicates if DL_{co} was determined by the single-breath (sb) or rebreathing (rb) technique. N is the number of species. In some cases, regression equations were calculated from data given in the reference. Units have been converted for equivalence, where necessary.

TABLE 6.27
Allometry of O₂ Consumption and Flux

Taxon	Status	Intercept	Slope	N	M_b Range
Mammals + 7 birds	Basal	10.2	0.734	19	0.016–3.833
Mammals	Rest	10.7	0.739	10	0.173–679
Mammals	Rest	11.6	0.76	—	—
Mammals	Std	9.8	0.756	12	0.021–600
Small mammals	Std	9.13	0.727	56	0.003–3.71
Fossorial mammals	Basal	5.62	0.557	16	0.021–0.63
Monotremes	Rest	4.72	0.79	6	0.73–4.9
Dasyurid marsupials	Basal	6.75	0.74	12	0.008–5.05
Marsupials	Std	6.94	0.737	8	0.014–32.49
Marsupials	Rest	14.4	0.74	29	0.01–5.1
Insectivores (>25 g)	Rest	21.4	0.73	12	0.03–0.89
Chiropterans	Rest	18.8	0.73	17	0.005–0.59
Edentates	Rest	14.6	0.77	12	1.6–4.7
Lagomorphs	Rest	23.7	0.72	11	0.07–5.9
Rodents	Rest	24.9	0.69	133	0.007–0.96
Higher primates	Rest	37.6	0.73	10	0.23–71
Carnivores	Rest	30.0	0.73	24	0.17–26
Artiodactyls and perissodactyls	Rest	34.6	0.73	19	13–665
Mammals	Max	100	0.845	45	0.007–677
Mammals	Max	107	0.854	19	0.020–469

(continued)

TABLE 6.27 (continued)
Allometry of O₂ Consumption and Flux

Taxon	Status	Intercept	Slope	N	M _b Range
Mammals	Max	128	0.856	33	0.007–469
Mammals	Max	115	0.809	22	0.007–263
Canids	Max	219	0.905	4	4.6–27.6
Mammals	Max	54.0	0.678	14	0.003–2.54
Mammals	Max	67.5	0.73	4	0.033–0.841

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Notes: Allometry of oxygen consumption and flux through the respiratory system (V_{O_2} mL [STPD] O₂ · min⁻¹) under resting (rest), basal (basal), standard (std), and maximal (max) conditions. V_{O_2} is related to body mass (M_b in kg) by the function. $V_{O_2} = a \cdot M_b^b$, where a is the intercept of the log-transformed linear regression, and b is the slope of the log-transformed linear regression. N is the number of species. In some cases, regression equations were calculated from data given in the reference. Units have been converted for equivalence, where necessary.

TABLE 6.28
Allometry of Blood Variables

Taxon	Intercept	Slope	N	M _b Range
Blood Volume (cm³ · kg⁻¹)				
Eutherians	65.6	1.02	—	—
Mammals	76	1.0	97	0.01–10 ³
Hematocrit (%)				
Mammals	45.8	-0.01	123	—
Oxygen Half-Saturation Pressure (P₉₀, Torr)				
Eutherians and marsupials	39.4	-0.03	89	—
Marsupials	32.1	-0.074	7	—
Small mammals	50.3	-0.054	17	0.021–6.35
Bohr Effect (Δlog P₉₀/ΔpH)				
Mammals (hemoglobin solution)	0.76	-0.0596	10	0.03–3140
Erythrocyte Carbonic Anhydrase (U/μL Erythrocyte* or U/100 μg Hemoglobin**)				
Mammals*	1.146	-0.107	15	0.006–601
Mammals**	0.695	-0.107	13	0.025–4000

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Notes: Variables are related to body mass (M_b , in kg) by the function: $Y = a \cdot M_b^b$, where Y is the variable, a is the intercept of the log-transformed linear regression, and b is the slope of the log-transformed linear regression. N is the number of species. In some cases, regression equations were calculated from data given in the reference. Units have been converted for equivalence, where necessary.

TABLE 6.29
Values of Standard (Basal) Oxygen Consumption for Representative Mammalian Species of Different Body Mass

Species	M _b (kg)	V _{O₂} std (mL [STPD] O ₂ · S ⁻¹ kg ⁻¹)
Horse (<i>Equus caballus</i>)	703	0.040
Ox (<i>Bos taurus</i>)	700	0.031
Pig (<i>Sus scrofa</i>)	122	0.048
Human (<i>Homo sapiens</i>)	71.5	0.058
Sheep (<i>Ovis aries</i>)	51.5	0.071
Goat (<i>Capra hircus</i>)	36	0.054
Dog (<i>Canis familiaris</i>)	13.2	0.026
Rabbit (<i>Oryctolagus cuniculus</i>)	4	0.183
Rhesus monkey (<i>Macaca mulatta</i>)	3.2	0.034
Cat (<i>Felis catus</i>)	3	0.122
Guinea pig (<i>Cavia porcellus</i>)	0.900	0.230
Rat (<i>Rattus norvegicus</i>)	0.375	0.247
Hamster (<i>Mesocricetus auratus</i>)	0.115	0.278
Gerbil (<i>Meriones unguiculatus</i>)	0.090	0.389
Mouse (<i>Mus musculus</i>)	0.030	0.528

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

TABLE 6.30
Allometric Equations for Respiratory Variables
in Mammals ($Y = aM^b$; M = mass in kg)

Variable (Y)	Coefficient (a)	Exponent (b)
Tidal volume (mL)	7.69	1.04
Dead space volume (mL)	2.8	0.96
	—	1.05
Lung volume	—	1.02
Total lung capacity (mL)	53.5	1.06
Vital capacity (mL)	56.7	1.03
Functional residual cap (mL)	24.1	1.13
Wet lung wt (g)	11.3	0.99
	7.72	1.03
Lung compliance (mL/cm H ₂ O)	2.10	1.08
Inflationary	1.94	1.08
Deflationary PoMax ^a 20	3.09	1.15
Deflationary PoMax ^a 8	2.2	1.09
Chest wall compliance	4.52	0.86
Inflationary	5.59	1.2
Deflationary PoMax 20	4.61	1.07
Deflationary PoMax 8	4.9	1.16
Respiratory system comp.	1.56	1.04
Inflationary	1.34	1.1
Deflationary PoMax 20	1.8	1.11
Deflationary PoMax 8	1.56	1.06
Ventilation (mL/min)	379	0.8
Oxygen consumption (V_{O_2})(mL/min)	11.6	0.76
Oxygen consumption (V_{O_2}) (mL/s)	0.188	0.75
V_{O_2} Max (mL/s)	1.92	0.809
$V\tau/T\tau$ (inspiratory flow)(mL/s)	13.5	0.74
Resistance (raw) (cm H ₂ O/L/s)	24.4	-0.70
Resistance (raw) (cm H ₂ O/mL/s)	0.078	-0.819
Reciprocal time const. (s ⁻¹)	8.97	-0.298
Upper airway R (cm H ₂ O/mL/s)	0.056	-0.702
Rec. time const. law + uaw ^b	5.272	-0.326
Frequency (min ⁻¹)	53.5	-0.26
	—	0.28

Source: Boggs, D.F., Comparative control of respiration, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a PoMax, maximum airway pressure.

^b law, lower airways; uaw, upper airways.

TABLE 6.31
Maximal Oxygen Consumption for Mammals

Species	M_b (kg)	$V_{O_{2max}}$ (mL [STPD] $O_2 \cdot s^{-1} \cdot kg^{-1}$)
Horse, thoroughbred (<i>Equus caballus</i>)	466	2.76
Horse, standardbred (<i>E. caballus</i>)	453	2.23
Ox, steer (<i>Bos taurus</i>)	449	0.85
Eland (<i>Taurotragus oryx</i>)	240	0.60
Zebu ox (<i>Bos indicus</i>)	232	0.49
Pony (<i>E. caballus</i>)	171	1.48
Hereford calf (<i>B. taurus</i>)	141	0.61
Waterbuck (<i>Kobus defassa</i>)	109.8	0.79
Pony (<i>E. caballus</i>)	105.0	1.61
Wildebeest (<i>Connochaetes taurinus</i>)	102.0	0.73
Human, untrained (<i>Homo sapiens</i>)	69.8	0.81
Human, trained (<i>H. sapiens</i>)	63.9	1.18
Goat (<i>Capra hircus</i>)	30.0	0.95
Lion (<i>Panthera leo</i>)	30.0	1.00
Dog (<i>Canis familiaris</i>)	28.0	2.29
Wolf (<i>Canis lupus</i>)	27.6	2.60
Dog (<i>C. familiaris</i>)	25.3	2.67
African sheep (<i>Ovis aries</i>)	21.8	0.78
Dog (<i>C. familiaris</i>)	21.0	2.64
African goat (<i>Capra hircus</i>)	20.9	0.87
Pig (<i>Sus scrofa</i>)	18.5	1.56
Coyote (<i>Canis latrans</i>)	12.4	3.10
Grant's gazelle (<i>Gazella granti</i>)	10.1	0.89
Red fox (<i>Vulpes vulpes</i>)	4.61	2.89
Blue fox (<i>Alopex lagopus</i>)	4.40	3.62
Dik-dik (<i>Madoqua kirkii</i>)	4.2	0.91
Spring hares (<i>Pedetes capensis</i>)	3.00	1.62
Genet cat (<i>Genetta tigrina</i>)	1.4	1.8
Banded mongoose (<i>Mungos mungo</i>)	1.14	1.9
Rat kangaroo (<i>Bettongia penicillata</i>)	1.10	2.95
Guinea pig (<i>Cavia porcellus</i>)	0.841	1.12
Dwarf mongoose (<i>Helogale pervula</i>)	0.58	2.1
White rat (<i>Rattus norvegicus</i>)	0.376	1.51
White rat, untrained (<i>R. norvegicus</i>)	—	1.21
White rat, trained (<i>R. norvegicus</i>)	—	1.56
White rat (<i>R. norvegicus</i>)	0.205	1.61
Hamster (<i>Mesocricetus auratus</i>)	0.100	1.97
Chipmunk (<i>Tamias striatus</i>)	0.0902	3.97
Merriam's chipmunk (<i>Eutamias merriami</i>)	0.075	1.96
Lemming (<i>Dicrostonyx groenlandicus</i>)	0.061	2.05
Mouse (<i>Mus musculus</i>)	0.033	2.89
Deer mouse (<i>Peromyscus maniculatus</i>)	—	2.95
European woodmouse (<i>Apodemus sylvaticus</i>)	0.020	4.4
Pygmy mouse (<i>Baiomys taylori</i>)	0.0072	4.36

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: Values of maximal oxygen consumption for mammals of different body mass measured with animals running on a motorized treadmill.

TABLE 6.32
Blood Respiratory Variables

Species	M_b (kg)	[Hb] (g · dL ⁻¹)	HCT (%)	O ₂ Capacity (mL [STPD] O ₂ · dL ⁻¹)	P ₅₀ (Torr)	Bohr Effect ($\Delta \log$ P ₅₀ / Δ pH)	Haldane Effect (mL [STPD] CO ₂ dL ⁻¹)	Temperature Coefficient ($\Delta \log$ P ₅₀ / Δ T)
African elephant (<i>Loxodonta africana</i> a.)	2000	15.3	42.7	20.5	23.2	-0.351	5.5	
Asian elephant (<i>Elephas maximus</i>)	1400	14.9	42	20.0	25.2	-0.351	5.5	
White rhinoceros (<i>Ceratotherium simum</i>)					20	-0.62		
Ox (<i>Bos taurus</i>)		11.5	40		31.5	-0.49		0.023
Horse (<i>Equus caballus</i>)		11.1	33.4		25.1	-0.45		0.023
Camel (<i>Camelus dromedarius</i>)		9.41	22.1	12.6	25.9			
Tapir (<i>Tapirus terrestris</i>)						-0.58		
Man (<i>Homo sapiens</i>)	67.0		44.8		26.5	-0.47		0.024
Bladdernose seal (<i>Cystophora cristata</i>)	50	26.4	63	36	24	-0.66	8.4	
Pronghorn antelope (<i>Antilocapra americana</i>)	38.4	16.8	42.7	22.5	28.3			
Orangutan, juvenile (<i>Pongo pygmaeus</i>)	34.6	12.9	40.9	17.4	24.4	-0.55		
Sheep (<i>Ovis aries</i>)	33.0		35.0		32.0	-0.49		
Chimpanzee (<i>Pan troglodytes</i>)	30.4	13.5	41.2	17.3	26.7	-0.462	4.7	
Pig (<i>Sus scrofa</i>)	30				35.7	-0.441		0.016
Goat (<i>Capra hircus</i>)	25.0		34.0		29.3	-0.48		
Dog (<i>Canis familiaris</i>)	20.0		40.0		29.0	-0.48		0.022
Gorilla, juvenile (<i>Gorilla gorilla</i>)	16.5	12.3	42.0	16.4	25.4	-0.48		
Kangaroo (<i>Macropus giganteus</i>)	15	18.6	53.0	24.9	27.5	-0.54		
Wallaby (<i>Macropus eugenii</i>)					32			
Baboons (<i>Papio anubis</i>)	10.36	11.7	37.1	15.4	37.2	-0.550	4.7	
Pigtail monkey (<i>Macaca nemestrina</i>)	9.50	12.6	42.0	16.6	36.7	-0.520	4.5	
Tasmanian devil (<i>Sarcophilus harrisii</i>)	8	20.1	47.0	26.9	41.2	-0.47		
Woodchuck (<i>Marmot monax</i>)	4.45	13.5	39.4		27.8	-0.72		
Rhesus monkey (<i>Macaca mulatta</i>)	3.94	12.9	41.5	16.9	35.2	-0.518	4.4	
Armadillo (<i>Dasypus novemcinctus</i>)	3.9	11.1	31	14.8	23.3	-0.55		
Pangolin (<i>Manis pentadactyla</i>)	3.6		36.5		25	-0.51		
Opossum (<i>Didelphis virginiana</i>)	3.40		31.5		38.7	-0.49		
Rabbit (<i>Oryctolagus cuniculus</i>)	3.10		35.0		30.0	-0.43		
Cat (<i>Felis domesticus</i>)	2.64		42		35.0	-0.50		
Echidna (<i>Tachyglossus setosus</i>)	2.1	17.6	48	21.9	21.3	-0.49		0.014
Platypus (<i>Ornithorhynchus anatinus</i>)	1.8	18.3	52	22.7	27.2	-0.56		0.014
Prairie dog (<i>Cynomys ludoricianus</i>)	1.28	15.1	47.3		22			
Muskrat (<i>Ondatra zibethica</i>)	0.97	13.3	36.2		26.1	-0.66		
Squirrel monkey (<i>Saimiri sciureus</i>)	0.95	14.3	42.0	17.7	35.5	-0.542	4.5	
Hedgehog (<i>Erinaceus europaeus</i>)	0.820		47.0	19.0	36	-0.49		0.017
Guinea pig (<i>Cavia porcellus</i>)	0.669		41.1		26.7	-0.48		
Ground squirrel (<i>Spermophilus beecheyi</i>)	0.598	15.1	48.7		26			
White rat (<i>Rattus norvegicus</i>)	0.555		41.9		36.0	-0.52		
Guinea pig (<i>Cavia porcellus</i>)	0.291	12.6			25.3			
Mole rat (<i>Spalax ehrenbergi</i>)	0.196	15.0	45.6		29.5	-0.53		
Bat (<i>Rousettus aegyptiacus</i>)	0.146	20.0	55		30.8	-0.55		
Golden hamster (<i>Mesocricetus auratus</i>)	0.135		42.4		26.0	-0.44		
Pocket gopher (<i>Thomomys bottae</i>)	0.135	17.1	46		33.3	-0.61		
Naked mole rat (<i>Heterocephalus glaber</i>)	0.106	13.6	46		23.3	-0.43		

TABLE 6.32 (continued)
Blood Respiratory Variables

Species	M_b (kg)	[Hb] (g · dL ⁻¹)	HCT (%)	O ₂ Capacity (mL [STPD] O ₂ · dL ⁻¹)	P ₅₀ (Torr)	Bohr Effect ($\Delta \log$ P ₅₀ /ΔpH)	Haldane Effect (mL [STPD] CO ₂ dL ⁻¹)	Temperature Coefficient ($\Delta \log$ P ₅₀ /ΔT)
Gerbil (<i>Meriones unguiculatus</i>)	0.078		31.2		28.5	-0.51		
Mouse (<i>Mus musculus</i>)	0.029		36.4		34.7	-0.50		
Bat (<i>Pipistrellus pipistrellus</i>)	0.005	24.4	61.5		36.6	-0.47		
Mole (<i>Talpa europaea</i>)			47.2	22.4	24	-0.47		
Shrew (<i>Crocidura russula</i>)			35.5	21.7	37	-0.63		

Source: Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Notes: Body mass is M_b ; hemoglobin concentration, [Hb]; hematocrit, Hct; O₂-carrying capacity of the blood, O₂ capacity; oxygen half-saturation pressure, P₅₀, and oxygen equilibrium curve, temperature coefficient.

TABLE 6.33
Normal Values of Blood Gases and Blood Buffering

Species	Body Weight (kg)	T _B (°C)	Pa _{CO₂} (Torr)	pH _a	Pa _{O₂} (Torr)	HCO ₃ ⁻ (mEq/L)	Δlog Pa _{CO₂} ΔpH
Mice	0.028	37.5	20.1			12.3	-1.75
Gerbil	0.078	36.4	31.2			19.0	-1.45
Hamster	0.135	35.8	45.1			27.5	-1.53
Rat	0.555	37.6	40.7	7.426	83.5	24.9	-1.48
Rat	0.531		33.1	7.425	87.1		
Rat	0.297		39.8	7.467	91	28.7	
Guinea pig	0.669	37.8	40.2	7.395	90	23.5	-1.43
Cat	2.64	38.8	29.4	7.384	102	18	-1.39
Cat		38.9	32.5	7.426	108	21	
Rabbit	3.1	38.8	32.8	7.388	86	21	
Porcupine	5.87	37.8	34.6	7.383	95	23	-1.35
Coatimundi	6	37.6	23.4	7.411	99	15	
Baboon	8–11		37.6	7.388	106		
Minipig	11–19		47	7.432	94.3	30.5	
Dog	20	38.3	41.6	7.386	93	23.2	-1.53
Goat	25	39	41.0	7.40	94.6	25.7	-1.30
Sheep	33	39	40.9	7.44	96	27.6	-1.35
Sheep	32–37	40	40.2	7.46	107		
Calf	45–73		38.7	7.340	85.7	20.6	
Calf			47.3	7.41	92	29	
Man	67	37	40.5	7.38	93	23.3	-1.55
Pony	176–204	38	39.6	7.429	90	25.6	
Horse	387–543			7.408		25.8	
Burrowers and Hibernators							
Pocket gopher			45	7.381		28.1	-2.67
Syrian hamster	0.142		52.3	7.419	70.9		
Hamster (<i>Cricetus</i>)		37	45.3	7.40		28.2	
Ground squirrels (13-lined)		38	55.9	7.44		36.8	
			47.7	7.4	65		
			52.4	7.418	75.3	33	
Echidna	3.4		53	7.429	60.5	33	
Woodchuck	4.5	37.3	48	7.357	72	25.7	-1.29

Source: Boggs, D.F., Comparative control of respiration, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

TABLE 6.34
Body Weight and Lung Volumes in Fischer-344 Rats at Various Ages^a

Parameter	3 Months	18 Months	27 Months
Body weight (g)	222 ± 61	334 ± 106	332 ± 71
Total lung capacity (TLC) (mL)	11.9 ± 1.7	13.9 ± 2.2	14.4 ± 1.9
TLC/body weight (mL/kg)	56 ± 8	42 ± 7	43 ± 6
Vital capacity (mL)	11.0 ± 1.8	13.4 ± 2.3	13.4 ± 1.7
Functional residual capacity (mL)	2.1 ± 0.3	1.7 ± 0.3	2.7 ± 0.4
Residual volume (RV) (mL)	1.0 ± 0.3	0.6 ± 0.2	1.1 ± 0.5
RV/TLC, (mL/mL)	0.08 ± 0.03	0.04 ± 0.01	0.07 ± 0.03

Sources: Adapted from Mauderly, J.L., *Exp. Aging Res.*, 8, 31, 1982; Sahebhami, H., Aging of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. I, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Values are means ± SD.

TABLE 6.35
Body Weight and Lung Volumes in Adult and Older Hamsters^a

Parameter	15 Weeks	65 Weeks	P Value
Body weight (g)	126 ± 12	125 ± 7	>0.20
Total lung capacity (mL)	9.6 ± 1.3	11.1 ± 1.0	<0.02
Vital capacity (mL)	6.9 ± 1.0	7.8 ± 0.9	<0.10
Functional residual capacity (mL)	3.5 ± 0.5	4.3 ± 0.3	<0.05
Residual volume (RV) (mL)	2.7 ± 0.60	3.3 ± 0.3	<0.05
RV/TLC (%)	28 ± 5	30 ± 5	>0.20

Sources: Adapted from Mauderly, J.L., *Exp. Aging Res.*, 5, 497, 1979; Sahebhami, H., Aging of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. II, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Values are means ± SD.

TABLE 6.36
Ventilatory Parameters in Fischer-344 Rats of Various Ages^a

Parameter	3 Months	18 Months	27 Months
Respiratory frequency (breath/min)	48 ± 6	54 ± 7	54 ± 6
Tidal volume (mL)	1.1 ± 0.3	1.5 ± 0.3	1.5 ± 0.3
Minute ventilation (mL/min)	54 ± 14	82 ± 23	82 ± 18
Body weight (mL/min/kg)	254 ± 48	251 ± 45	252 ± 52

Sources: Adapted from Mauderly, J.L., *Exp. Aging Res.*, 8, 31, 1982; Sahebhami, H., Aging of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. I, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Values are means ± SD.

TABLE 6.37
Ventilatory Parameters in Hamsters at Various Ages^a

Parameter	15 Weeks	65 Weeks
Respiratory frequency (breath/min)	24 ± 2.7	25 ± 3.9
Tidal volume (mL)	1.2 ± 0.2	1.1 ± 0.2
Minute volume (mL/min)	27.8 ± 3.3	28.1 ± 4.0

Sources: Adapted from Mauderly, J.L., *Exp. Aging Res.*, 5, 497, 1979; Sahebhami, H., Aging of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. II, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Values are means ± SD.

TABLE 6.38
Lung Volumes (% TLC)^a at Transpulmonary Pressures of 10 (V_{10}) and 5 cm H₂O (V_5) for Various Young Adult Mammals

Species	(% TLC)		Conditions
	V_{10}	V_5	
Sci whale	68	45	Excised, room temperature, peak $P_L = 25$ cm H ₂ O
Horse	86	66	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 30$ cm H ₂ O
Humans	63	53	<i>In vivo</i> , body temperature, sitting, peak $P_L = 35$ cm H ₂ O
Sheep	78	66	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 30$ cm H ₂ O
Goat	81	51	Excised, room temperature, peak $P_L = 35$ cm H ₂ O
Dog	77	61	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 30$ cm H ₂ O
Monkey	93	82	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 30$ cm H ₂ O
Cat	93	71	Excised, room temperature, peak $P_L = 23$ cm H ₂ O
Rabbit	84	70	Excised, room temperature, peak $P_L = 30$ cm H ₂ O
Guinea pig	79	56	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 28$ cm H ₂ O
Rat	80	52	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 25$ cm H ₂ O
Hamster	84	64	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 25$ cm H ₂ O
White mouse	81	61	<i>In vivo</i> , anesthetized, body temperature, peak $P_L = 38$ cm H ₂ O

Source: Lai, Y.-L., Comparative ventilation of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. I, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: TLC, total lung capacity.

TABLE 6.39
Morphometric Values in Sprague–Dawley Rats of Various Ages^a

Parameter	4 Months	8 Months	18 Months
V_L body weight (mL/kg) ^b	21.7 ± 1.0	30.9 ± 1.5	38.4 ± 2.8
Lm (μm) ^b	54 ± 2	71 ± 2	87 ± 7
ISA (cm^2)	5.571 ± 445^c	7.979 ± 318	8.733 ± 721

Sources: Adapted from Johanson, W.G., Jr. and Pierce, A.K., *J. Clin. Invest.*, 52, 2921, 1973; Sahebhami, H., Aging of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission. All rights reserved.

^a Values are means \pm SEM. V_L postfixation lung volume; Lm, mean chord length; ISA, internal surface area.

^b Significantly different among groups.

^c Significantly different compared with other groups.

BRONCHOALVEOLAR LAVAGE FLUID (BALF) (TABLES 6.40 THROUGH 6.43)

TABLE 6.40
Normal Cytology of BALF (% of Total Cells)

Animal	Macrophages	Neutro	EOS	Lymph
Rat, mouse, rabbit, Syrian hamster	95	<1	<1	<1
Guinea pig	90	—	10	—
Rabbit	95	<1	<1	4
Dog	85	5	5	5
Sheep	70	5	5	15
Horse	83	5	<1	10
Monkey	89	—	—	10
Human (nonsmoker)	88	<1	<1	10

Source: Reproduced from Henderson, R.F., Bronchoalveolar lavage: A tool for assessing the health status of the lung, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, Chapter 15, 1989. With permission. All rights reserved.

Note: BALF, bronchoalveolar lavage fluid; Neutro, neutrophil; EOS, eosinophils; Lymph, lymphocytes.

TABLE 6.41
Normal Biochemical Content of BALF, X (SE)^a

Animal	<i>n</i>	LDH (mIU/mL)	Alkaline Phosphatase (mIU/mL)	Acid Phosphatase (mIU/mL)	β -Glucuronidase (mIU/mL)	Protein (mg/mL)
Rat	240–280	109 (2)	53 (1)	2.4 (0.1)	0.34 (0.02)	0.39 (0.02)
Mouse	45–95	233 (13)	2.5 (0.2)	7.5 (0.8)	0.53 (0.08)	0.82 (0.07)
Guinea pig	6	69 (26)	5.7 (1.6)	2.5 (0.2)	0.65 (0.12)	0.13 (0.03)
Syrian hamster	6	72 (7)	3.6 (1.0)	2.0 (0.1)	0.57 (0.09)	0.37 (0.03)
Rabbit	6	27 (6)	8.5 (4.4)	5.3 (0.5)	0.37 (0.02)	0.44 (0.10)
Dog	4–12	134 (25)	22 (5)	1.4 (0.1)	0.30 (0.04)	0.35 (0.18)
Chimpanzee	5	51 (12)	53 (3)	—	—	0.01 (9.01)

Source: Reproduced from Henderson, R.F., Bronchoalveolar lavage: A tool for assessing the health status of the lung, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, Chapter 15, 1989. With permission. All rights reserved.

^a Values are normalized per milliliter of lung volume washed.

TABLE 6.42
Relative Proportions of Immunocompetent Cell Populations Obtained by Bronchoalveolar Lavage

Species	Bronchoalveolar Cells (%)			Lymphocyte (%)	
	Macrophages	Lymphocytes	Granulocytes	T	B
Human	78–91	9–20	1–3	47	15–17
Monkey	90–91	3–6	3–6	62	4
Dog	59–75	22–39	0–9	—	—
Swine	60–70	30–33	0–6	—	—
Guinea pig	50–80	12–50	2–19	68–76	10–20
Rabbit	84–98	2–16	0	—	—
Hamster	89	3	10	—	—
Mouse	45–96	3–39	1–6	—	—
Rat	93	2	5	50	12

Sources: Adapted from McDermott, M.R. et al., *Int. Rev. Exp. Pathol.*, 23, 47, 1982; Murray, M.J. and Driscoll, K.E., Immunology of the respiratory system, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

TABLE 6.43
Lymphocyte Subpopulations Observed in Bronchoalveolar Lavage Fluid of Lung Tissue

Species: Source ^a	T Lymphocytes (%)				B Lymphocytes (%)
	Total	Helper	Suppressor	Helper/Suppressor	
Human					
BALF	73	46	25	1.8	NR
	72	46	28	1.7	9
	63	45	25	1.9	5.3
	66	48	27	1.8	NR
Tissue	40	NR ^b	NR	—	10
Rat					
BALF	32	29	NR	—	NR
	50	31	25	1.2	12.3
Tissue	90	50	40	1.2	10
	62	29	NR	—	15
	51	26	24	1.1	25
	44	16	19	0.8	10
	59	28	36	0.8	12
Mouse					
Tissue	38	13	6	2.2	23

Source: Murray, M.J. and Driscoll, K.E., Immunology of the respiratory system, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a BALF, bronchoalveolar lavage fluid. Tissue lymphocyte populations were obtained by enzyme digestions and/or mechanical disruption techniques.

^b NR, not reported.

PULMONARY DEPOSITION AND CLEARANCE
(TABLES 6.44 THROUGH 6.47; FIGURES 6.9
THROUGH 6.12)

TABLE 6.44

Lung and Alveolar Macrophage (AM) Parameters as They May Relate to *In Vivo* Particle Uptake

	Mammalian Species						
	Mouse	Hamster	Rat	Guinea Pig	Rabbit	Dog	Human
Average body weight (g)	42	122	380	430	2,600	16,000	74,000
Lung volume (mL)	1.45	3.9	10.9	13	112	1,320	4,340
Lung surface area (m ²)	0.125	0.28	0.66	0.91	3.3	52	143
Alveolar diameter (μm)	47	60	70	65	88	126	219
Calculated no. of alveoli (millions) ^a	18	25	43	69	135	1,040	950
Lavagable no. of AM animal (millions)	0.36	2.0	1.6	1.4	12	3,876	6,500
	0.38	4.2	3.0	3.4	17		
	0.53	5.1	3.8	4.7	28		
	0.73	5.8	7.6		43		
	1.36	6.3	8.3		49		
Average	0.67	4.7	4.9	3.2	30	3,876	6,500
Calculated AMs alveolus	0.037	0.19	0.11	0.046	0.22	3.7	6.8
Area patrolled by each AM (μm ²)	190,000	60,000	140,000	280,000	110,000	13,400	22,000
<i>In vivo</i> gold colloid uptake by AM, $T_{1/2}$ (h)	7.1	0.8	4.2		3.2	Corr. coeff. with area = $r^2 = 0.99$	

Source: Valberg, P.A. and Blanchard, J.D., Pulmonary macrophage physiology: Origin, motility, endocytosis, Parent, R.A., Ed., in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Number of alveoli = alveolar surface area/ π (alveolar diameter).²

TABLE 6.45

Tracheal Mucociliary Clearance

Species	Mucous Velocity ^a (mm/min)
Mouse	+
Rat	1.9 ± 0.7
	5.1 ± 3.0
	5.9 ± 2.5
Ferret	+
	18.2 ± 5.1
	10.7 ± 3.7
Guinea pig	2.7 ± 1.4
Rabbit	3.2 ± 1.1
	+

(continued)

TABLE 6.45 (continued)
Tracheal Mucociliary Clearance

Species	Mucous Velocity ^a (mm/min)
Chicken	*
Cat	2.5 ± 0.8
Dog	21.6 ± 5.0
	9.8 ± 2.1
	19.2 ± 1.6
	7.5 ± 3.7
	14.5 ± 6.3
Baboon	+
Sheep	17.3 ± 6.2
	10.5 ± 2.9
Pig	*
Cow	*
Donkey	14.7 ± 3.8
Horse	16.6 ± 2.4
	17.8 ± 5.1
Human	3.6 ± 1.5
	5.5 ± 0.4
	5.1 ± 2.9
	11.5 ± 4.7
	10.1 ± 3.5
	21.5 ± 5.5
	15.5 ± 1.7

Source: Wolff, R.K., Mucociliary function, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Note: *, Transport studied but no velocity given; +, Inhalation study, clearance measured but no tracheal velocities given.

^a Mean ± SD.

TABLE 6.46
Nasal Mucociliary Clearance

Species	Velocity ^a (mm/min)
Rat	2.3 ± 0.8
Dog	3.7 ± 0.9
Man	5.2 ± 2.3
	5.5 ± 3.2
	5.3 (0.5–23.6)
	8.4 ± 4.8
	6.8 ± 5.1
	7 ± 4

Source: Wolff, R.K., Mucociliary function, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Mean ± SD.

TABLE 6.47
Comparative Pulmonary Clearance Data for Relatively Insoluble Particles Inhaled
by Laboratory Animals and Humans

Species	Aerosol Matrix	Particle Size		Pulmonary Burden ^a				Study Duration (Days)
		μm	Measure	P ₁	T ₁	P ₂	T ₂	
Mouse	FAP	0.7	AMAD	0.93	34	0.07	146	850
	FAP	1.5	AMAD	0.93	35	0.07	171	850
	FAP	2.8	AMAD	0.93	36	0.07	201	850
	Ru Oxide	0.38	CMD	0.88	28	0.12	230	490
	Pu Oxide	0.2	CMD	0.86	20	0.14	460	525
Rat	Diesel soot	0.12	MMAD	0.37	6	0.63	80	330
	FAP	1.2	CMD	0.62	20	0.38	180	492
	FAP	0.7	AMAD	0.91	34	0.09	173	850
	FAP	1.5	AMAD	0.91	35	0.09	210	850
	FAP	2.8	AMAD	0.91	36	0.09	258	850
	Latex	3.0	CMD	0.39	18	0.61	63	190
	Pu Oxide	<1.0	CMD	0.20	20	0.80	180	350
	Pu Oxide	2.5	AMAD	0.75	30	0.25	250	800
	U ₃ O ₈	~1–2	CMD	0.67	20	0.33	500	768
	FAP	2.0	AMAD	0.22	29	0.78	385	1100
	Diesel soot	0.12	MMAD			1.00	>2000	432
Guinea pig	Latex	3.0	CMD			1.00	83	190
	Coal dust	2.4	MMAD			1.00	1000	160
	Coal dust	1.9	MMAD			1.00	~700	301–392
Dog	Ce Oxide	0.09–1.4	MMD			1.00	>570	140
	FAP	2.1–2.3	AMAD	0.09	13	0.91	440	181
	FAP	0.7	AMAD	0.15	20	0.85	257	850
	FAP	1.5	AMAD	0.15	21	0.85	341	850
	FAP	2.8	AMAD	0.15	21	0.85	485	850
	Nb Oxide	1.6–2.5	AMAD			1.00	>300	128
	Pu Oxide	1–5	CMD			1.00	1500	280
	Pu Oxide	4.3	MMD			1.00	300	300
	Pu Oxide	1.1–4.9	MMAD		~1		400	468
	Pu Oxide	0.1–0.65	CMD	0.10	200	0.90	1000	~4000
	Pu Oxide	0.72	AMAD	0.10	3.9	0.90	680	730
	Pu Oxide	1.4	AMAD	0.32	87	0.68	1400	730
	Pu Oxide	2.8	AMAD	0.22	32	0.78	1800	730
	Tantalum	4.0	AMAD	0.40	1.9	0.60	860	155
	U ₃ O ₈	0.3	CMD	0.47	4.5	0.53	120	127
	Zr Oxide	2.0	AMAD			1.0	340	128
	Pu Oxide	2.06	CMAD			1.0	500–900	200
	Pu Oxide	1.6	AMAD			1.0	770–1100	990
Human	FAP	1	CMD	0.14	40	0.86	350	372–533
	FAP	4	CMD	0.27	50	0.73	670	372–533
	Latex	3.6	CMD	0.27	30	0.73	296	~480
	Latex	5	CMD	0.42	0.5	0.58	150–300	160
	Pu Oxide	0.3	MMD			1.00	240	300
	Graphite and PuO ₂	6	AMAD			1.00	240–290	566
	Pu Oxide	<4–5	CMD			1.00	1000	427
	Th Oxide	<4–5	CMD			1.00	300–400	427
	Teflon	4.1	CMD	0.30	4.5–45	0.60	200–2500	300
	Zr Oxide	2.0	AMAD			1.00	224	261

Source: Snipes, M.B., *Crit.Rev. Toxicol.*, 20, 174, 1989. With permission.

Notes: FAP, fused aluminosilicate particles; AMAD, activity median aerodynamic diameter; MMAD, mass median aerodynamic diameter; CMD, count median diameter; MMD, mass median diameter. Some aerosols were monodisperse, most were poly-disperse, with GSD in the range of 1.5–4. Clearance half-times are approximations for biological clearance, the net result of dissolution–absorption processes and physical clearance processes. In some examples, the original data were subjected to a computer curvefit procedure to derive the values for P and T .

^a Pulmonary burden $P_1 e^{(\ln 2)/T_1} + P_2 e^{(\ln 2)/T_2}$, where P_1 and P_2 equal fractions of the initial pulmonary deposition, T_1 and T_2 are equal retention half-times in days, and t equals days after exposure.

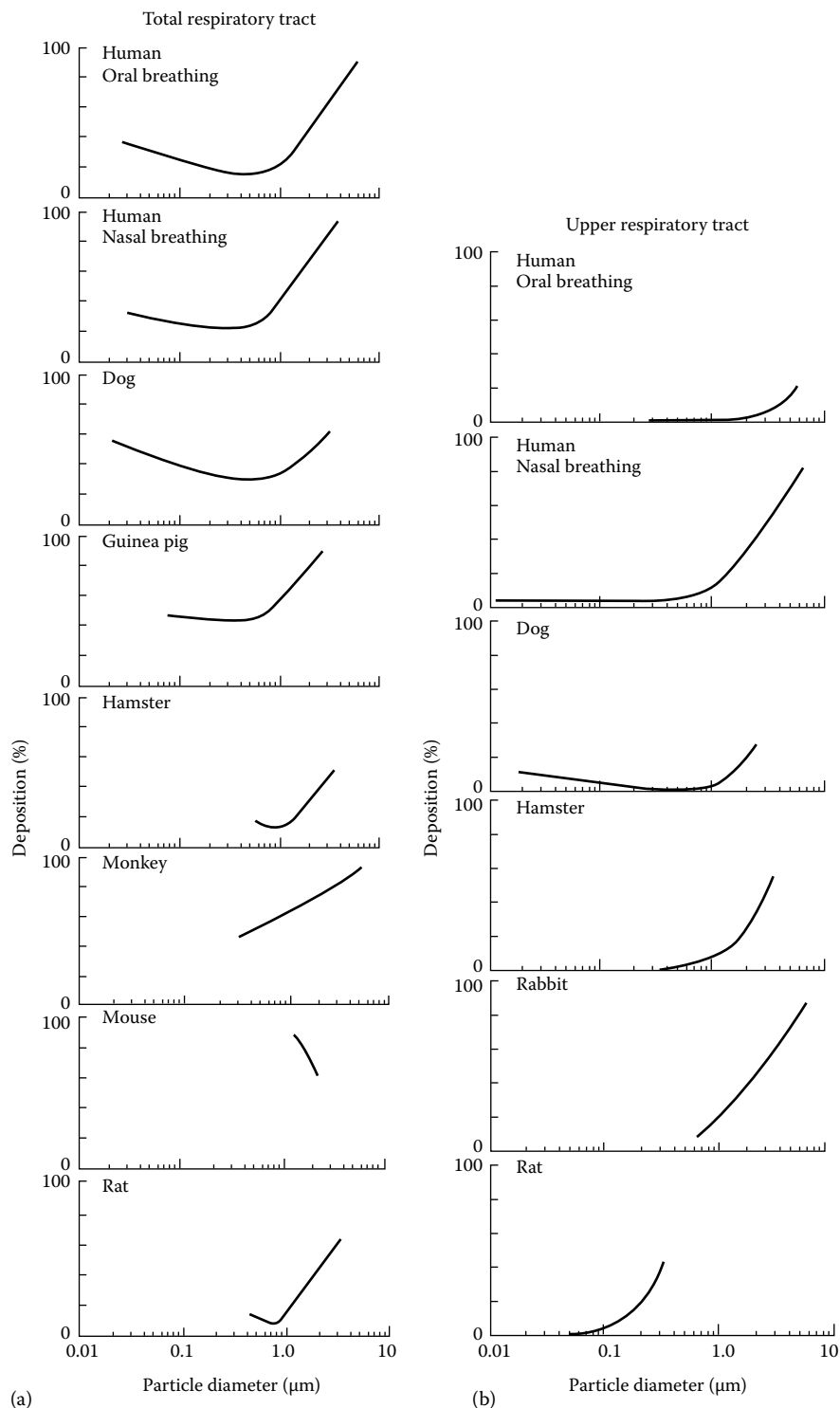


FIGURE 6.9 Particle deposition efficiency in experimental animals as a function of particle size for (a) total respiratory tract, (b) upper respiratory tract. Each curve represents an eye fit through mean values (or centers of ranges) of the data. Similar curves for humans are shown for comparison. Particle diameters are aerodynamic for those $\geq 0.5 \mu\text{m}$ and diffusion equivalent for those $< 0.5 \mu\text{m}$. (Reproduced from Schlesinger, R.B., in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, Chapter 6, p. 208, 1989. With permission. All rights reserved.)

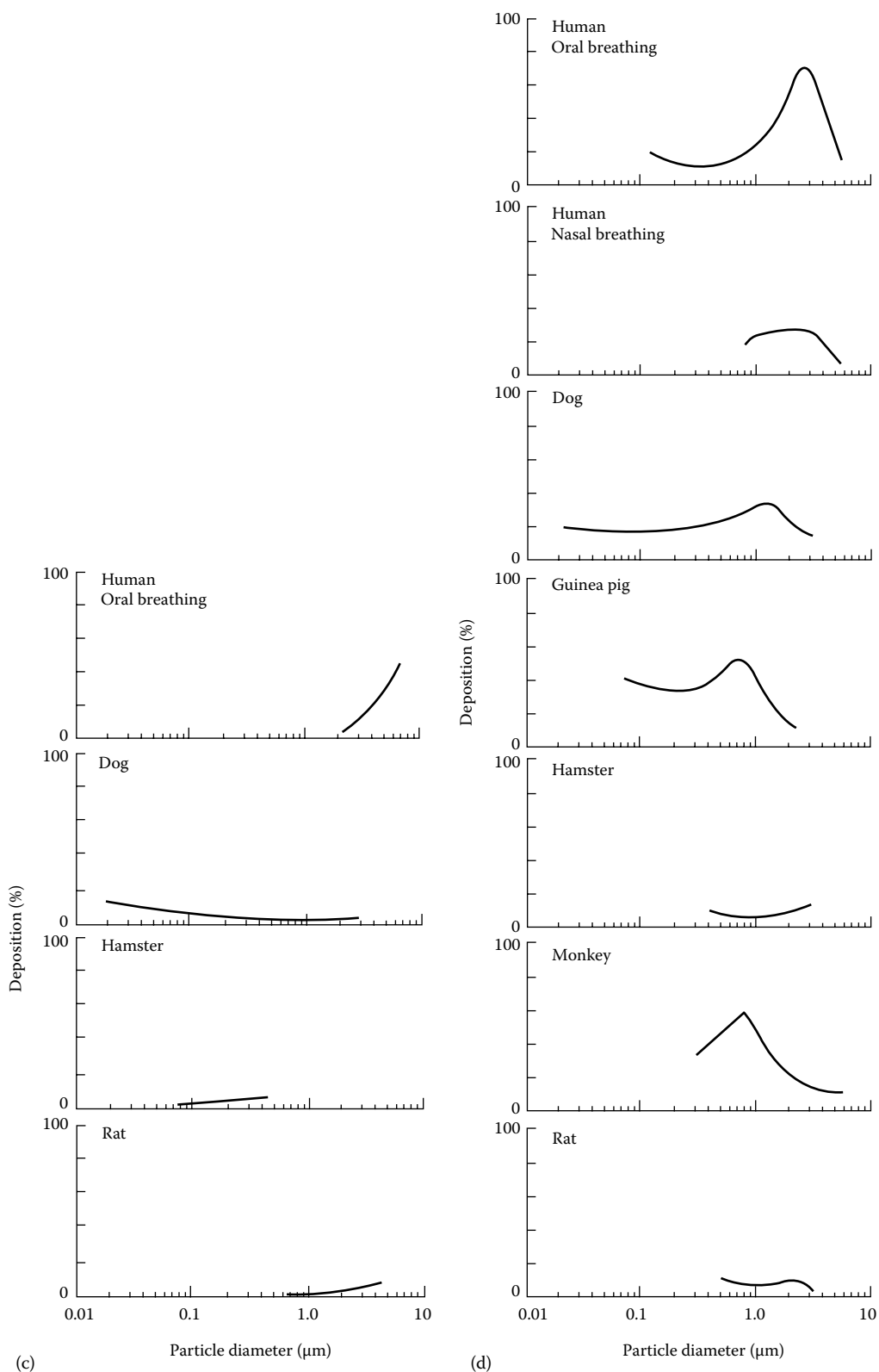


FIGURE 6.9 (continued) Particle deposition efficiency in experimental animals as a function of particle size for (c) tracheobronchial tree, and (d) pulmonary region. Each curve represents an eye fit through mean values (or centers of ranges) of the data. Similar curves for humans are shown for comparison. Particle diameters are aerodynamic for those $\geq 0.5 \mu\text{m}$ and diffusion equivalent for those $< 0.5 \mu\text{m}$. (Reproduced from Schlesinger, R.B., in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, Chapter 6, p. 208, 1989. With permission. All rights reserved.)

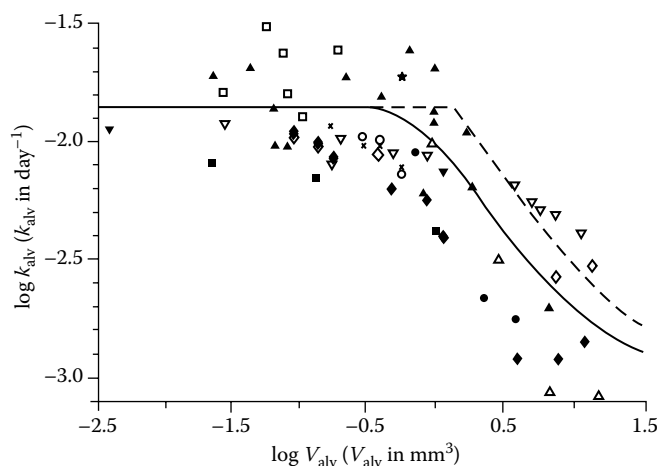


FIGURE 6.10 Alveolar clearance rate (k_{alv}) as a function of total particle volume in the lung, with data from different investigators using different insoluble particles. (From Stöber, W. et al., Approaches to modeling disposition of inhaled particles and fibers in the lung, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.)

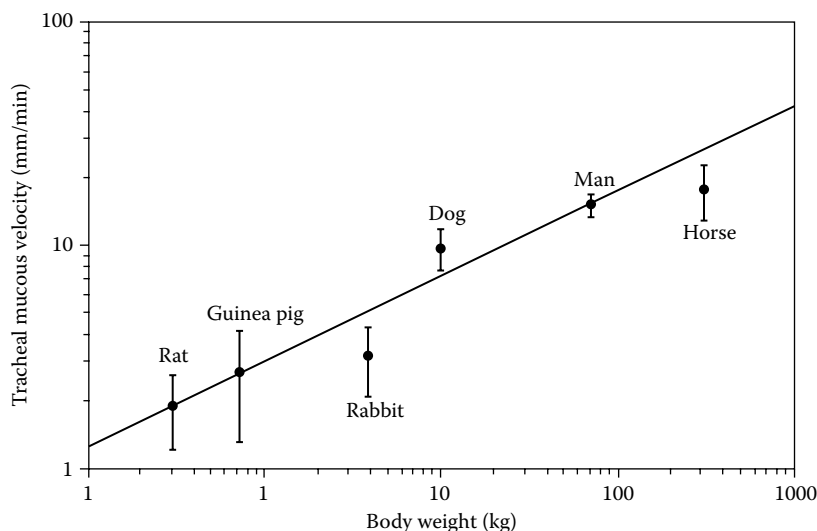


FIGURE 6.11 Tracheal mucous velocities in a log-log plot vs. the body weight of a range of species. The same techniques of intratracheal instillation of $^{99\text{m}}\text{Tc}$ -MAA were used in all cases. The function $\text{TMV} = 3.0 (\text{BW})^{0.39}$ defines the relation between tracheal mucous velocity and body weight, with a correlation of 0.94. (From Wolff, R.K., Mucociliary function, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.)

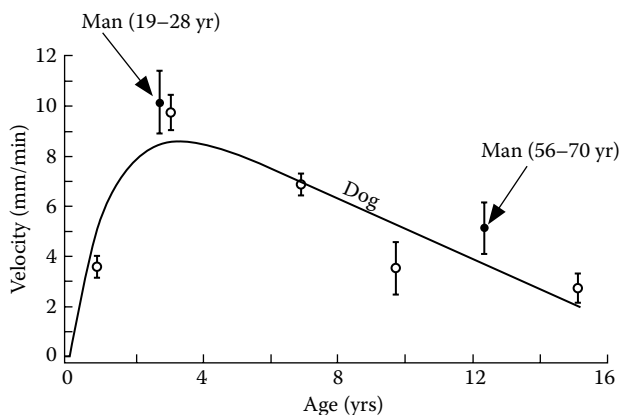


FIGURE 6.12 Tracheal mucous velocity (mean \pm SE) is shown for beagle dogs (M) vs. age. The fitted function that describes this relation is $V(t) = 11[1 - \exp(-0.9t)] - 0.6t$. The available data for humans (v) are also shown after transforming for age. (From Wolff, R.K., Mucociliary function, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.)

**PULMONARY TOXICITY (TABLES 6.48
THROUGH 6.55; FIGURES 6.13 THROUGH 6.15)**

TABLE 6.48
Slopes of Ventilatory Responses to Carbon Dioxide

Species	Slope
Rat (Sprague–Dawley)	6.6
Rat (Wistar)	6.4
Rabbit	23.0
Cat	12.8
Porcupine	33.0
Woodchuck	12.0
Coatimundi	23.0
Baboon	34
Dog	38.3
Dog	13.4
Goat	32.7
Human	28
Pony	38
Weddell seal	16.7
Harbor seal	7.7
Harbor seal	23.12
Hooded seal	6.52
Harp seal	11.1

Source: Boggs, D.F., Comparative control of respiration, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

TABLE 6.49
Species Comparison of Lung Function Response after Exposure to Air Pollutants

Toxicant	Species	mg/m ³	Time	<i>f</i>	<i>V</i> _T	<i>V</i> _g	<i>R</i>	<i>C</i>
Ozone	GP	2.1	2 h	↑	↓	—	↑	NR
	Rabbit	23.5	~35 h	↑	↓	↑	↑	↓
	Rat	0.7	2 h	↑	↓	—	—	—
	Dog	~2.0	2 h	↑	↓	↑	NR	↓
	Cat	0.5	4.6 h	Fix	Fix	Fix	↑	↓
Sulfur dioxide	GP	0.84	1 h	↑	↓	NR	↑	↓
	Monkey	13.4	78 wk	—	—	—	—	—
	Dog	13.4	225 day	—	—	—	↑	↓
	Sheep	13.1	4 h	—	—	—	—	—
	Mouse	44.5	10 min	↓	NR	NR	NR	NR
	Ferret	1308	12 wk	↓	—	—	—	—
	Cat	52.4	30 min	Fix	Fix	Fix	↑	↓
Sulfuric acid	GP	0.7	1 h	—	—	—	↑	↓
	Rat	6.4	14 weeks	—	—	—	—	—
	Dog	5.0	4 h	—	—	—	—	—
	Monkey	4.8	78 weeks	↑	—	—	—	—
	Donkey	1.5	1 h	NR	NR	NR	—	—
	Rabbit	0.3	1 year	NR	NR	NR	—	NR

(continued)

TABLE 6.49 (continued)
Species Comparison of Lung Function Response after Exposure to Air Pollutants

Toxicant	Species	mg/m ³	Time	<i>f</i>	<i>V_T</i>	<i>V_E</i>	<i>R</i>	<i>C</i>
Nitrogen dioxide	Rat	3.8	2 years	↑	↑	—	—	—
	Monkey	9.4	90 day	↑	↑	—	—	—
	GP	9.8	4 h	↑	↓	—	—	—
	Rabbit	15.0	12 weeks	NR	NR	NR	↑	NR
	Cat	19.2	12 months	NR	NR	NR	↑	↓
	Sheep	28	4 h	NR	NR	NR	↑	—
	Hamster	38.4	14 months	NR	NR	NR	↑	—
	Dog	69.5	4 h	↑	↓	NR	NR	NR
Formaldehyde	GP	4.7	8 h	↓	↑	NR	↑	NR
	Rat	38.9	10 min	↓	↓	↓	NR	NR
	Mice	6.0	10 min	↓	↑	↓	NR	NR

Source: Costa, D.L. et al., Interpretations and limitations of pulmonary function testing in small laboratory animals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

Notes: *f*, Frequency of breathing; *V_T*, tidal volume; *V_E*, minute ventilation; *R*, resistance; *C*, compliance; GP, guinea pig; ↑, increase; ↓, decrease; —, no change observed; NR, not reported; Fix, mechanically ventilated.

TABLE 6.50
Lung Dysfunction after Toxicant Exposure

Toxicant	Species	Functional Variable ^a
SO ₂ + ZnO	Guinea pig	↓ LV, ↓ DL _{CO}
(NH ₄) ₂ SO ₄	Guinea pig	↑ LV, ↓ N ₂ slope
Coal dust	Rat	↓ LV, ↑ FEF
Diesel exhaust	Rat	↑ LV, ↑ FEF
Diesel exhaust	Rat	DL _{CO} , ↓ C _{rs} , ↑/↓ N ₂ slope
O ₃	Rat	—VP, ↑ R _{sw}
O ₃	Rat	↑ VP
O ₃	Rat	↑ LV, —C _{rs} , —N ₂ slope, — DL _{CO}
O ₃	Rat	↑ LV, ↑ VP, ↓ PaO ₂
O ₃	Rat	↑ LV, ↑ VP, ↓ FEF, — DL _{CO}
Acrolein	Rat	↑ LV, ↑/↓ C _{rs} , ↓ N ₂ slope, DL _{CO} , ↑ ↓ FEF
MIC	Rat	↓ PK flow
		↓ FEF 50, 25, +10
SiO ₂	Rat	↓ LV, ↓ C _{rs} , ↓ N ₂ slope, ↓ DL _{CO} , ↓ FEF
Cl ₂	Rat	↑ LV, ↑ C _{rs} , —N ₂ slope, — DL _{CO} , —FEF
MIC	Rat	↓ DL _{CO} , ↓ C _{rs} , ↓ N ₂ slope
Cd	Rat	↓ LV, ↓ C _{rs} , ↓ N ₂ slope, ↓ DL _{CO} , ↑ ↓ FEF
O ₂	Rat	↑/↓ LV, ↓ C _{rs} , ↓ DL _{CO} , ↓ FEF
Oil fog	Rat	↓ LV, DL _{CO} , —C _{rs} , —N ₂ slope
Volcanic ash/SO ₂	Rat	—LV, —DL _{CO} , —C _{rs} , —N ₂ slope
SiO ₂	Rat	↓ LV, ↓ DL _{CO}
NO ₂	Mouse	—LV, DL _{CO} , ↓ C _{rs} , ↑ N ₂ slope
O ₃	Rabbit	↑ LV, ↓ FEF
O ₃ , SO ₂ olefin	Hamster	—LV, ↓ N ₂ slope, ↑ DL _{CO}

Source: Costa, D.L. et al., Interpretations and limitations of pulmonary function testing in small laboratory animals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992. With permission.

^a Functional variables: LV, lung volume; DL_{CO}, diffusion capacity for carbon monoxide; FEF, forced expiratory flow; C_{rs}, respiratory system compliance; R_{sw}, airway resistance; VP, volume–pressure curve; ↑, increase; ↓, decrease, — no change.

TABLE 6.51
Agents Causing Lung Tumors in Laboratory Animals
after Inhalation Exposure

Organic chemicals

Gases

Bis(chloromethyl)ether
 Bromoethane (ethyl bromide)
 1,3-Butadiene
 1,2-Dibromo-3-chloropropane
 1,2-Dibromoethane
 Dimethyl sulfate
 1,2-Epoxybutane
 Ethylene oxide
 Methylene chloride
 3-Nitro-3-hexene
 1,2-Propylene oxide
 Tetrachloroethylene
 Tetranitromethane
 Urethane
 Vinyl chloride

Particles

Benzo(a)pyrene
 Polyurethane dust

Inorganic compounds

Metallic

Antimony compounds
 Beryllium compounds
 Cadmium chloride
 Chromium dioxide
 Nickel compounds
 Titanium compounds

Nonmetallic

Asbestos fibers
 Zeolite fibers
 Ceramic aluminosilicate fibers
 Kelvar aramid fibers
 Silica
 Oil shale dust
 Quartz
 Volcanic ash

Radionuclides

Alpha-emitting radionuclide particles
 Beta-emitting radionuclide particles
 Radon and its decay products

Complex mixture

Cigarette smoke
 Diesel engine exhaust
 Gasoline engine exhaust
 Coal tar aerosols
 Artificial smog

Source: Hahn, F.F., Chronic inhalation bioassays for respiratory tract carcinogenesis, in *Toxicology of the Lung*, Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.

TABLE 6.52
Carcinogenic Agents Causally Associated with Human
Lung or Pleural Cancer

Industrial processes

Aluminum production
 Coal gasification
 Coke production
 Hematite mining, underground with exposure to radon
 Iron and steel founding
 Painter, occupational exposure
 Rubber industry

Chemicals and groups of chemicals for which exposure has been primarily occupational

Asbestos
Bis(chloromethyl)ether
 Chromium compounds, hexavalent
 Coal tars
 Coal tar pitches
 Mustard gas
 Nickel and nickel compounds
 Soots
 Talc containing asbestiform fibers
 Vinyl chloride

Environmental agents and cultural risk factors

Erionite
 Radon and its decay products
 Tobacco smoke

Source: Hahn, F.F., Chronic inhalation bioassays for respiratory tract carcinogenesis, in *Toxicology of the Lung*, Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993. With permission.

Note: Based on database of IARC Monographs Program.

TABLE 6.53
Effects of Inhaled Toxicants on Mucociliary Clearance

Toxicant	Exposure Concentration	Exposure Duration	Animal Species	Region Examined ^a	Response ^b
Ozone (O ₃)	0.5 ppm	2 h	Sheep	T	NE
	1 ppm	2 h	Sheep	T	↓
	0.4–1.2 ppm	4 h	Rat	B	↓ At 0.8 ppm
	0.1–0.6 ppm	2 h	Rabbit	B	↓ At 0.6 ppm
	0.62–1.25 ppm	4 h	Mouse	B	NE
	0.25, 0.6 ppm	2 h/day, 14 days	Rabbit	B	NE
	0.2, 0.4 ppm	2 h (with exercise)	Human	B	↑
Nitrogen dioxide(NO ₂)	7.5, 15 ppm	2 h	Sheep	T	↓ At 15 ppm
	0.3–10 ppm	2 h	Rabbit	B	NE
	6 ppm	7 days/wk 6 wk	Rat	T	↓
	0.3, 1 ppm	2 h/day, 14 days	Rabbit	B	NE
Sulfur dioxide (SO ₂)	1, 5, 25 ppm	6 h	Human	N	↓ At 5, 25 ppm
	5 ppm	3 h	Human	B	NE
	5 ppm	2 h (with exercise)	Human	B	↑
	20 ppm	4 h	Rat	B	↓
	1 ppm	1 yr	Dog	T	↓
Formaldehyde (HCHO)	20 ppm	4 h	Rat	PB	↓
	0.5, 15 ppm	6 h/days, 5 days/week, 3 weeks	Rat	N	↓ At _6 ppm
Carbon particles	50 mg/m ³	Few minutes	Human	TB	↑
Diesel exhaust particles	0.4–0.5 mg/m ³	0.5 h	Sheep	T	NE
	17 mg/m ³	4 h	Rat	T	↓
	2 mg/m ³	7 h/day, 5 days/week, 6 months	Rat	B	NE
	0.35–7 mg/m ³	7 h/day, 5 days/week, 30 months	Rat	T	NE
Sulfuric acid (H ₂ SO ₄)	0.4–0.5 mg/m ³	0.5 h	Sheep	T	NE
	14 mg/m ³ (0.1 μm)	0.3 h	Sheep	T	NE
	4 mg/m ³ (0.1 μm)	4 h	Sheep	T	NE
	1, 5 mg/m ³ (0.3 μm)	1 h	Dog	T	NE
	0.2–1.4 mg/m ³ (0.4 μm)	1 h	Donkey	T, B	NE on T, ↓ B persistent in 2 of 4 animals
	0.1–10 mg/m ³ (0.5 μm)	1 h	Human	T, B	NE on T, ↑↓ B (depending on concentration)
	0.5, 1 mg/m ³ (0.9 μm)	1 h	Dog	T	↓
	3.6 mg/m ³ (1 μm)	4 h	Rat	B	NE
	1.5 mg/m ³ (0.6 μm)	4 h	Mouse	B	NE
	15 mg/m ³ (3.2 μm)	4 h	Mouse	B	↓
	0.1–2.2 mg/m ³ (0.3 μm)	1 h	Rabbit	B	↑ At low concentration
				B	↓ At high concentration
	0.25 mg/m ³ (0.3 μm)	1 h/day, 5 days/week, 1 year	Rabbit	B	↓
	0.25–0.5 mg/m ³ (0.3 μm)	1 h/day, 5 days/week, 4 weeks	Rabbit	B	↑
	0.1 mg/m ³ (0.5 μm)	1 h/day, 5 days/week, 6 months	Donkey	B	↓
Ammonium bisulfate (NH ₄ HSO ₃)	0.6–1.7 mg/m ³ (0.4 μm)	1 h	Rabbit	B	↓ Only at 1.7 mg/m ³
	1 mg/m ³ (0.1 μm)	4 h	Sheep	T	NE
Ammonium sulfate [(NH ₄) ₂ SO ₄]	2 mg/m ³ (0.4 μm)	1 h	Rabbit	B	NE
	0.3–3 mg/m ³ (0.4 μm)	1 h	Donkey	T, B	NE
	3.6 mg/m ³ (0.4 μm)	4 h	Rat	B	NE
	1.1 mg/m ³ (0.1 μm)	4 h	Sheep	T	NE

Source: Schlesinger, R.B., *Crit. Rev. Toxicol.*, 20, 297, 1990. With permission.

^a N, nasal passages; B, bronchial tree; T, trachea.

^b NNE, no effect; ↑, acceleration of clearance or mucus transport rate or time; ↓, retardation of clearance or mucus transport rate or time. Median particle size.

TABLE 6.54
Effects of Inhaled Toxicants on Clearance from the Respiratory Region of the Lungs

Toxicant	Exposure Concentration	Exposure Duration	Animal Species	Response ^a
Ozone (O ₃)	0.1–1.2 ppm	2 h	Rabbit	↑ At 0.1 ppm, ↓ at 1.2 ppm
	0.1, 0.6 ppm	2 h/day, 14 days	Rabbit	↑
	0.4–1 ppm	4 h	Rat	↑ At 0.8, 1 ppm
	0.5 ppm	16 h/day, 2 or 5 months	Rabbit	NE
Nitrogen dioxide (NO ₂)	0.3–10 ppm	2 h	Rabbit	↑
	0.3, 1 ppm	2 h/day, 14 days	Rabbit	↑
	1, 10 ppm	2 h/day, 14 days	Rabbit	↑
	30, 60 ppm	5 h/day, 5 days/week, 2 weeks	Mouse	↓ At 60 ppm
	3–24 ppm	7 h/day, 5 days/week, 2–3 weeks	Rat	↑ At low conc. × time values; ↓ At high conc. × time values
Formaldehyde (HCHO)	20 ppm	4 h	Rat	NE
Sulfur dioxide (SO ₂)	10 ppm	16 h/day, 20 weeks	Rabbit	↑
	0.1–20 ppm	7 h/day, 5 days/week, 2–5 weeks	Rat	↑ At low conc. × time values, ↓ At high conc. × time values
Sulfuric acid (H ₂ SO ₄)	1 mg/m ³ (0.3 μm)	1 h	Rabbit	↑
	3.6 mg/m ³ (1 μm)	4 h	Rat	↓
	0.25 mg/m ³ (0.3 μm)	1 h/day, 5 days/wk up to 240 days	Rabbit	↑
Ammonium sulfate [(NH ₄) ₂ SO ₄]	3.6 mg/m ²	4 h	Rat	NE
Lead	76, 161 mg/m ³	7 mo	Rabbit	NE
Silica	21.1 mg/m ³	5 h/day, 4 days/week, 1 year	Rat	↓
Chromium [Cr(VI)]	0.2 mg/m ³	Continuous, 42 days	Rat	↓
Diesel exhaust particles	0.2–4.1 mg/m ³	7 h/day, 5 days/week, 18 weeks	Rat	↓ At 4.1 mg/m ³
	0.35, 3.5, 7 mg/m ³	7 h/day, 5 days/week, 30 months	Rat	↓ At ≥3.5 mg/m ³
	4 mg/m ³	95 h/week, 19 months	Rat, Hamster	↓
Carbon black	7 mg/m ³	20 h/day, 7 week, 1–6 days/week	Rat	↓

Source: Schlesinger, R.B., *Crit. Rev. Toxicol.*, 20, 297, 1990. With permission.

^a NE, no effect; ↑, acceleration of clearance rate or time; ↓, retardation of clearance rate or time; conc., concentration.

TABLE 6.55
Effects of Inhaled Toxicants on the Phagocytic Activity of Alveolar Macrophages

Toxicant	Exposure Concentration ^a	Exposure Duration	Animal Species	Response ^b
Ozone (O ₃)	1.2 ppm	2 h	Rabbit	↓
	0.1 ppm	2 h/day, 13 days	Rabbit	↓
	0.8 ppm	20 days	Rat	↑
	2.5 ppm	5 h	Rat	↓
	0.3–0.5 ppm	3 h	Rabbit	↓
	0.8 ppm	4 h	Rat	↓
Nitrogen dioxide (NO ₂)	10.25 ppm	24 h	Rat	↓ At 25 ppm
	0.3, 1 ppm	2 h/day, 13 days	Rabbit	↓ At 0.3, ↑ at 1 ppm
	0.5 ppm	3–5 h/day, 5 days/week, 1–3 months	Mouse	↓ At 2 mo
Sulfur dioxide (SO ₂)	1, 5, 10, 20 ppm	24 h	Rat	↑ At ≥5 ppm
Ethylene (CH ₂ O)	10, 20 ppm	24 h	Rat	↓ At 20 ppm
Chromium [Cr(VI)]	0.050 mg/m ³	28 days	Rat	↑
	0.025, 0.050, 0.200 mg/m ³	90 days, 6 h/day, 5 days/week, 4–6 weeks	Rat	↑ At 0.025–0.050 mg/m ³ , ↓ At 0.200 mg/m ³
Calcium chloride (CaCl ₂), Copper chloride (CuCl ₂), Cobalt chloride (CoCl ₂)	0.4–0.6 mg/m ³	6 h/day, 5 days/week, 4–6 weeks	Rabbit	NE
Cadmium (Cd)	1.5, 5.0 mg/m ³	0.5 h	Rat	↑ At 1.5 mg/m ³ , ↓ at 5 mg/m ³
Nickel dust (Ni)	0.13 mg/m ³	5 h/day, 5 days/week, 4–8 months	Rabbit	NE
Diesel exhaust particles	0.25, 1.5, 6 mg/m ³	Up to 12 months	Guinea pig, rat	↓

Source: Schlesinger, R.B., *Crit. Rev. Toxicol.*, 20, 297, 1990. With permission.

^a *In vivo* exposures.

^b ↓, depression of phagocytic activity; ↑, enhancement of phagocytic activity; NE, no effect.

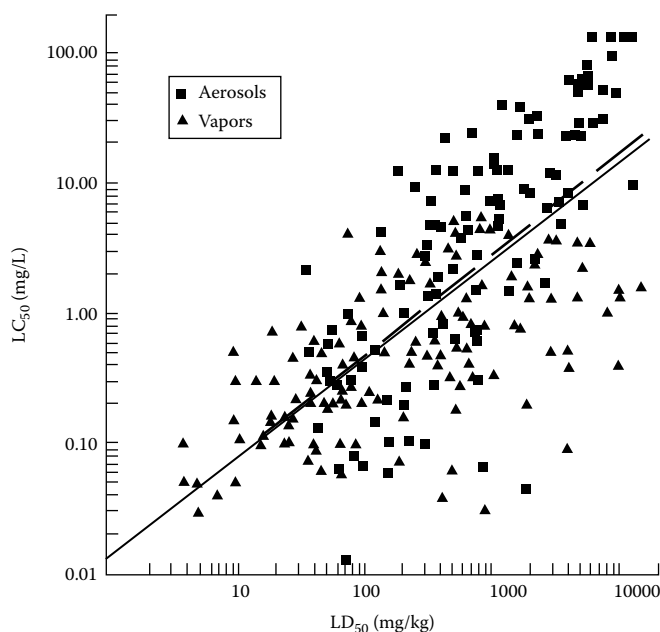


FIGURE 6.13 Double-logarithmic correlation of inhalation LC₅₀ and oral LD₅₀. Solid line, least-squares regression; broken line, nonparametric regression. (From Klimisch, H.-J. et al., *Regul. Toxicol. Pharmacol.*, 7, 21, 1987. With permission.)

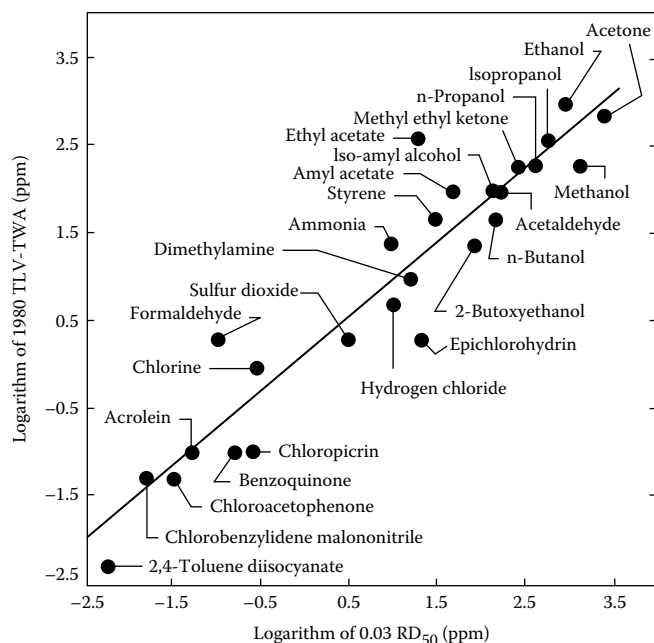


FIGURE 6.14 Correlation between time-weighted average threshold limit values (ACGIH) and RD_{50} values determined in mice for 26 irritants. (Compiled from the data from Kane, L.E. et al., *Am. Ind. Hyg. Assoc. J.*, 40, 207, 1979; Figure reprinted from Parent, R.A., *Comparative Biology of the Normal Lung*, CRC Press, Boca Raton, FL, 1992. With permission.)

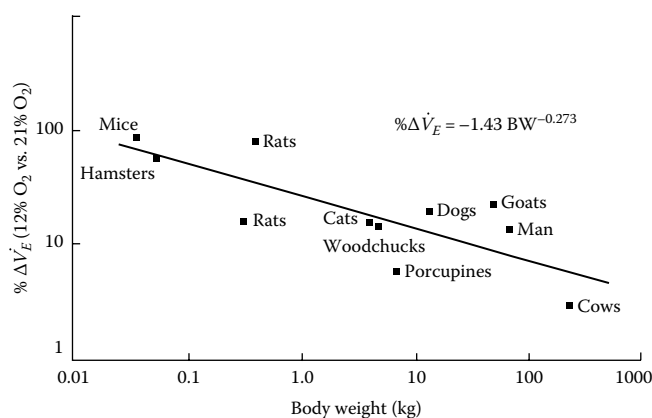


FIGURE 6.15 Ventilatory response to 12% inspired O_2 (or PI_{O_2} of 90 Torr) in 10 species over a wide range of body size. (From Boggs, D.F. and Tenny, S.M., *Respir. Physiol.*, 58, 245, 1984. With permission.)

RESPIRATORY TRACT METABOLISM (TABLES 6.56 THROUGH 6.58)

TABLE 6.56
Some Xenobiotic Metabolizing Enzymes in the Nasal Cavity

Enzyme	Test Reaction or Other Method for Detection	Test System	Notes
15-Lipoxygenase P-450	Arachidonic acid metabolism Diethylnitrosamine deethylase	Human nasal cells Human nasal respiratory tissue microsomes	Nasal epithelial cells more active than bronchial cells Nasal activity per nmol P-450 10–25 times that of liver
P-450	Five dealkylases	Human nasal respiratory tissue microsomes	HMPA and aminopyrine best substrates; ethoxycoumarin and ethoxyresorufin next best substrates; pentoxyresorufin poor substrate
Epoxide hydrolase	Safrole oxide hydrolase	Human nasal respiratory tissue homogenate	Activity higher than that in rats
Glutathione S-transferase	1-Chloro-2, 4-dinitrobenzene conjugation	Human nasal respiratory tissue homogenate	Activity higher than that in rats
DT-diaphorase	Dichlorophenol-indophenol metabolism	Human nasal respiratory tissue homogenate	Activity much less than that in rats
UDP-glucuronyl transferase	1-Naphthol conjugation	Human nasal respiratory tissue homogenate	Absent in humans; present in rats
NADPH-cytochrome C-reductase	Cytochrome C reduction	Human nasal respiratory tissue homogenate	Activity about 25% that of rat nasal mucosa
Rhodanese	Metabolism of cyanide to thiocyanate	Human nasal respiratory tissue homogenate	Activity in nonsmokers twofold higher than that in smokers
P-450PB-B; NADPH-cytochrome P-450 reductase	Immunohistochemistry	Male Holtzman rat olfactory and respiratory tissues, Bowman's glands, and seromucous glands	P-450PB-B is homologous with IIB1; Bowman's glands and apex of olfactory epithelial cells contained high concentrations of reductase
P-450c	Immunocytochemistry	Male Alp/Apk rat olfactory epithelium; Bowman's glands	Homologous with P-450IA1; not induced by phenobarbital, clofibrate, or β -naphthoflavone
P-450 β NF-B P-450PB-B P-450PCN-E	Immunohistochemistry	Male Holtzman rat olfactory epithelium; Bowman's glands, and seromucous glands	P-450 β NF-B (homologous with IA1) present in olfactory tissue at a higher level than in respiratory tissue; consistent with this, aryl hydrocarbon hydroxylase activity; PB-B (IIB1) intensely stained both tissues; PCN-E (IIIA) stained less intensely, but about equally in both tissues; apical portions of epithelial cells and subepithelial glands stained relatively intensely
P-450d	Induction of encoding mRNA	S-D rat olfactory tissue microsomes	P-450d (IA2) but not P-450c (IA1) was induced to detectable levels
P-450olf1	cDNA library probe and sequence analysis	S-D rat olfactory tissue microsomes	Termed IIG1; rabbit form may be P-450NMb; olfactory tissue specific
P-450olf2 P-450IIE1	Immunoblots Immunohistochemistry	Wistar rat olfactory tissue Male F344 rat	Homologous with IIA family Glands in lamina propria heavily labeled; olfactory sustentacular cells apically labeled; ciliated cells of respiratory epithelium and nonsecretory cells of transitional epithelium labeled; luminal surface of olfactory epithelium in vomeral nasal organ labeled
Aromatase and 5 α -reductase	Testosterone metabolism to estradiol and dihydrotestosterone	Measled S-D rat olfactory epithelium	Castration decreased estradiol production; activity restored by testosterone replacement
FAD-containing mono-oxygenase	Dimethylamine and N,N-dimethylaniline metabolism	Male F344 rat olfactory mucosa microsomes	Dimethylaniline apparently metabolized only by FAD-MO; dimethylamine metabolized by P-450, as well
Aldehyde dehydrogenase; formaldehyde dehydrogenase	Formaldehyde and acetaldehyde dehydrogenation; histochemistry	Male F344 rat respiratory and olfactory mucosa	Multiple forms; formaldehyde dehydrogenase most abundant in olfactory mucosa; epithelial cell cytoplasm and olfactory sensory cell nuclei; Bowman's and seromucous glands weakly positive; acetaldehyde dehydrogenase most abundant in respiratory mucosa; present in Bowman's glands and olfactory basal cells; absent from sensory cells and sustentacular cells
Carboxylesterase	Ester hydrolysis; histochemistry	F344 rat nasal tissue	k_m values ranged from 1 to 35 mM; V_{max} from 0.03 to 0.06; present in all nasal cells except olfactory neurons
Carbonic anhydrase	Histochemistry	Rat olfactory tissue	Present in receptor cells; absent in sustentacular cells

TABLE 6.56 (continued)
Some Xenobiotic Metabolizing Enzymes in the Nasal Cavity

Enzyme	Test Reaction or Other Method for Detection	Test System	Notes
Epoxide hydrolase; UDP-glucuronyl transferase; glutathione S-transferase forms B, C, and E	Styrene oxide; 7-hydroxycoumarin; styrene oxide; immunohistochemistry	Male F344 or Holtzman rat nasal tissue homogenates	Epoxide hydrolase probably form A; GSH-T B, C, and E probably forms 5,5, 1,1, 3,3, respectively; GSH-T form C—which metabolizes ΔE^5 -androstene-3, 17-dione—was at highest levels
Rhodanese	Cyanide metabolism to thiocyanate; immunohistochemistry	F344 rat nasal mucosa	Highest in apical portion of olfactory epithelium; absent from receptor cells; negligible in Bowman's glands; present in respiratory epithelium also
P-4503a, P-450 form 2, P-450 form 4, P-450 form 5	Immunochemistry; immunoblot; enzyme assays	Male New Zealand white rabbit nasal mucosa	Homologues IIE; Forms 2, 4, 5, by homology, are also termed IIB1, 1A2, and IVB1, respectively; forms 2 and 5 occur in both respiratory and olfactory tissues; form 4 found in olfactory tissue only; form 6 (homologous with IA1) absent in all tissues
FAD-containing mono-oxygenase	Immunoblot	Male New Zealand white rabbit olfactory and respiratory mucus	Approximately equal amounts in respiratory and olfactory tissues
P-450 form NMa, P-450 form NMb, P-450 form 2, P-450 form 3a, P-450 form 3b, P-450 form 4, P-450 form 6	Immunochemistry; testosterone metabolism; HMPA and phenacetin metabolism	Male New Zealand white rabbit nasal respiratory and olfactory epithelium	Only form 2 (IIB1) found in respiratory tissue; NMa very active for HMPA and phenacetin dealkylation; only 3% of liver P-450 is NMa; NMb (IIG1) occurs only in olfactory tissue; forms 3a and 4 are homologous with IIE1 and IA2, respectively; forms 3b and 6 (homologous to IIC3 and IA1, respectively) absent in nasal tissue
Carboxylesterase	Ester hydrolysis	Male and female New Zealand white rabbit nasal mucosa	Less activity than for mice, rats, or dogs. Activities in both mucosae similar to that in liver
P-450	ρ -Nitroanisole demethylase; aniline hydroxylase; carbon monoxide difference spectra	CD1 mouse nasal tissue microsomes	Compared to dog, rabbit, guinea pig, rat, and Syrian hamster, aniline better substrate in mouse than in any other species except Syrian hamsters
P-450; NADPH-cytochrome C-reductase	7-Ethoxycoumarin deethylase; carbon monoxide difference spectrum; cytochrome C-reductase	MFI mouse olfactory epithelium	Very high nasal activities relative to those in liver; activities higher in males than in females
Carboxylesterases	Ethylene glycol monomethylether acetate	Male and female B6C3F ₁ /Crl Br mouse nasal mucosa homogenates	Mouse activity greater than that of rats
Carboxylesterases	ρ -Nitrophenyl butyrate hydrolysis; histochemistry	B6C3F ₁ mouse; both sexes; respiratory and olfactory tissues	Also examined rats; mouse and rat activity (V/k_m) similar; olfactory activity fivefold that of respiratory; V_{max} olfactory 0.5 μ mol/min/mg protein, k_m 20–25 mM
P-450	ρ -Nitroanisole, O-demethylase, and aniline hydroxylase; carbon monoxide difference spectra	Syrian hamster nasal tissue microsomes	Very high activities relative to dog, rabbit, guinea pig, rat, and mouse; values (in nmol P-450 per mg protein): olfactory —0.36, respiratory—0.13, trachea—0.26, liver—1.10
Arylhydrocarbon oxidases/hydroxylase	Benzo(a)pyrene metabolism <i>in vivo</i> ; olfactory and respiratory tissue microsomal activity	Syrian hamster nasal cavity	Also examined <i>in vitro</i> metabolism in 5 nonnasal tissues; olfactory tissue highest (4000 pmol/g tissue/h); metabolites <i>in vivo</i> included tetrols, diols, quinones, oxides, and phenols
P-450; NADPH cytochrome C-reductase; cytochrome b5	7-Ethoxycoumarin and ethoxyresorufin dealkylase; hexobarbitone oxidase; aniline hydroxylase; cytochrome C-reductase; difference spectra	Syrian hamster olfactory tissue	Very high activities compared to those in rats and female mice; olfactory activities (but not P-450 content) higher than in liver
Carboxylesterases	Ester hydrolysis of acetate esters and lactones; ethyl acetate uptake <i>in vivo</i>	Syrian hamster olfactory nasal cavity	Hamster nasal activity much higher than rat or rabbit nasal activity with amyl acetate, but not with β -butyrolactone; ester uptake in hamster nose sensitive to enzyme inhibition (63%–90%)
Alcohol dehydrogenase	Propanol metabolism <i>in vivo</i>	Syrian hamster tissue homogenates	k_m is 0.1 mM; V_{max} is 4 nmol/mg protein per min, 36 nmol/nose per min

Sources: Schlesinger, R.B. et al., Disposition of inhaled toxicants, in *Handbook of Human Toxicology*, Massaro, E.J., Ed., CRC Press, Boca Raton, FL, Chapter 12, pp. 493–550, 1997. With permission. Originally adapted from Dahl, A.R. and Hadley, W.M., *Toxicology*, 21, 345, 1991.

TABLE 6.57
Summary of P-450 Isozymes Reported in the Rat
and Rabbit Nasal Cavities

Isozyme	Alternate Name		Nasal Tissue	
	Rat	Rabbit	Rat	Rabbit
IA1	β NF-B	Form 6	Respiratory and olfactory	Absent
IA2	ISF-G,d	Form 4	Olfactory	Olfactory
IIA	olf2	—	Olfactory	Not reported
IIB1	PB-B,b	Form 2	Respiratory and olfactory	Respiratory and olfactory
IIC3	—	3b	Not reported	Absent
IIE1	j	3a,P450ALC	Respiratory and olfactory	Olfactory
IIG1	olf1	P-450LM3c	Respiratory and olfactory	Not reported
IIIA	PCN-E			
IVB1	Form 5	Form 5	Not reported	Respiratory and olfactory

Sources: Schlesinger, R.B. et al., Disposition of inhaled toxicants, in *Handbook of Human Toxicology*, Massaro, E.J., Ed., CRC Press, Boca Raton, FL, Chapter 12, pp. 493–550, 1997. With permission. Originally adapted from Dahl, A.R. and Hadley, W.M., *Toxicology*, 21, 345, 1991.

TABLE 6.58
Some P-450 Isozymes Reported in Lungs of Various Species

	Isozyme	Comments
Mouse	1A1	Induced in type II cells
	1A2	Induced in type II cells and endothelial cells
	2B1	Constitutive in type II cells and Clara cells
	2B2	Constitutive in type II cells and Clara cells
	4B1	Rabbit form activates ipomeanol and 2-aminofluorene
	“mN”	In Clara cells, metabolizes naphthalene
	“m50b”	In Clara cells, major naphthalene-metabolizing enzyme
Rat	1A1	Induced in bronchial epithelium, Clara cells, and type II cells
	2A3	Absent in rat liver
	2B1	Constitutive at highest levels in Clara cells
	2E1	Induced by hyperoxygen
	3A1/2	Induced in bronchial epithelium, Clara cells, and type II cells
	4B1	Absent in rat liver
	“FI”	Constitutive; induced by O ₃
Rabbit	“FII”	Cross reacts with rat anti-2B1; constitutive; induced by O ₃
	1A1	Highly inducible; occurs in endothelial cells without reductase
	2B1	With 4B1, accounts for 80% of uninduced P-450 in lung
	2E1, 2E2	2E1, 5% that of liver; 2E2, 2.5% that of liver
	4A4	P-450 prostaglandin ω -hydroxylase; occurs only in pregnant rabbits or after induction by progesterone
Hamster	4B1	Activates 2-aminofluorene and ipomeanol
	“MC”	Possibly IA1; highly inducible
	2B	Along with reductase, absent from mesothelium in adults
Human	4B	Present in mesothelium in adult
	1A1	Inducibility related to smoking and lung cancer
	2E1	Racial differences in 2E1 polymorphisms
	2F1	Ethoxycoumarin and pentoxiresorufin and 3-methylindole are substrates
	4B1	Unlike rabbit form, does not activate 2-aminofluorene

Sources: Schlesinger, R.B. et al., Disposition of inhaled toxicants, in *Handbook of Human Toxicology*, Massaro, E.J., Ed., CRC Press, Boca Raton, FL, Chapter 12, pp. 493–550, 1997. With permission. Originally adapted from Dahl, A.R. and Hadley, W.M., *Toxicology*, 21, 345, 1991.

EXPOSURES

Inhalation exposure in toxicology studies can be delivered by a variety of methods with system selection typically dependent on the physical characteristics of the test article, intended clinical treatment regimen, and animal species. The most common delivery methods are Whole-Body Exposure (WBE), Snout-Only (or Nose-Only) Exposure, Face-Mask Exposure, and Oropharyngeal (OP) Exposure. These methods will be described below. Less commonly used delivery methods are Head-Only Exposure, Intratracheal Dosing, and Intranasal Dosing. These latter methods will not be described in detail later.

As the name implies, Whole-Body Exposure (WBE) involves placement of the animals (both small animal species such as mice, rats, and rabbits, and less commonly, large animal species such as dogs, pigs, and primates) in an exposure chamber and delivery of the intended treatment into the containment volume. In addition to inhalation delivery, animals exposed by this route receive incidental exposure by a variety of other routes, primarily by ingestion during grooming activity during and after exposure and also by ocular exposure and dermal absorption. In many instances, the combined dose by the incidental routes substantially exceeds the inhalation dose. Administration by the WBE route is also associated with substantially increased

contamination containment issues due to the body burden of test article available for re-entrainment after animals are returned to their cages following exposure. Due to this limitation and risk, WBE methods are generally confined to studies that aim to identify occupational health and safety exposure limits for industrial and agricultural chemicals. Even for these groups of chemicals, WBE is generally confined to those test articles that are primarily or wholly present in the gas or vapor phase.

The alternative inhalation administration is most commonly referred to as snout-only (or nose-only) exposure, which for small animal species close restraint in a tube and for large animal species aerosol delivery to a mask or oropharyngeal tube. Exposure by these methods is designed to minimize dermal exposure (confined to the snout or muzzle), to minimize incidental exposure routes, and ensure delivery of aerosol to the respiratory tract. Even within snout-only exposure techniques, two distinct delivery methods have evolved, each of which has its own advantages and disadvantages. These two methods are referred to as Flow-Through and Directed-Flow (also commonly called Flow-Past). Snout-only delivery is frequently employed for the testing of industrial and agricultural chemicals but is almost universally employed for the testing of pharmaceutical products (Tables 6.59 and 6.60; Figure 6.16).

TABLE 6.59
Comparison of Snout-Only/Mask/OP Exposures versus Whole-Body Exposures

Snout-Only/Mask/OP	Whole-Body
Exposure by location of the animal on a rigid system or delivery of aerosol via flexible pipes.	Free moving animal in a cage that is immersed in the test atmosphere.
Animals are individually restrained in exposure tubes/slings/chairs.	Animals are individually held in cages. Commonly used for reproductive toxicity studies due to lower stress relative to restraint in tubes in snout-only delivery.
Flexibility in a number of animals attached to exposure system. Can remove animals during exposure without affecting others (important if toxicokinetic bleeding is required).	Impractical to remove individual animals during exposure as have to stop dosing whole group.
View of restrained animals may be limited.	Generally, a clearer view of animals allowing observations of behaviors.
Considerable effort may be required to load and unload animals and maintain animals during exposure.	Minimal animal management required during exposure.
Habituation/acclimatization to the method of restraint preferable—typically 3–5 days (rodents) and up to 21 days (large animals).	Habituation/acclimatization to the method of exposure not required.
Minimal deposition—mostly only on snout.	Deposition on whole body—on fur.
Impractical to provide water during exposure.	Water may be provided during exposure.
Directed air flow exposure systems minimally operated at the calculated RMV of the animals on the system. More commonly operated at a multiple (at least 2×) of the combined RMV of exposed animals.	The animals should be exposed in inhalation systems designed to sustain a dynamic air flow of at least 10 air changes per hour to ensure CO ₂ , H ₂ O, and O ₂ remain within appropriate concentration levels. The internal chamber temperature should be maintained within the acceptable range for the exposed species.
Internal chamber volume is of limited importance.	To ensure stability of a chamber atmosphere and maximize the animal's exposure to the test article, the total volume of the test animals should not exceed 5% of the volume of the test chamber.

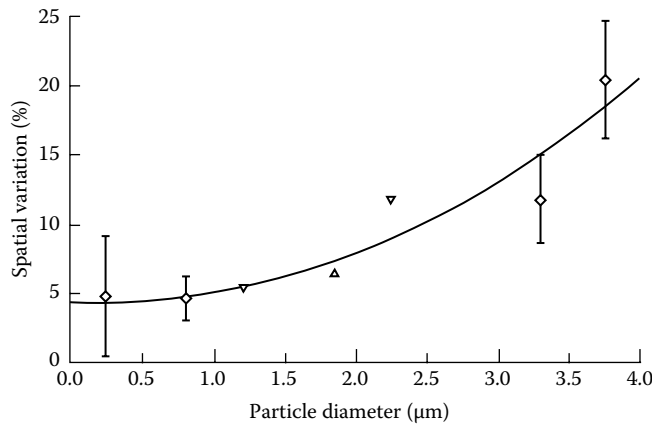


FIGURE 6.16 Spatial variations of aerosol concentrations in Hazelton 2000 exposure chambers as a function of aerosol particle size. Data are mean and standard deviation. (Reproduced from Cheng, Y.-S. and Moss, O.R., Inhalation exposure systems, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, Chapter 1, p. 50, 1989. With permission. All rights reserved.)

TABLE 6.60
Ammonia Concentrations in an Inhalation Chamber

Animal Loading (%)	Chamber Air Flow (L/min)	No. of Air Changes per Hour	Hour of Sample (ppm NH ₃ ± SE)		
			2	4	6
1	13	8	0.38 ± 0.08	0.48 ± 0.07	0.46 ± 0.13
1	26	16	0.20 ± 0.01	0.24 ± 0.02	0.45 ± 0.06
1	40	24	0.19 ± 0.04	0.24 ± 0.05	0.22 ± 0.03
3.1	13	8	0.84 ± 0.14	1.13 ± 0.14	1.11 ± 0.27
3.1	26	16	0.60 ± 0.09	1.04 ± 0.23	1.60 ± 0.22
3.1	40	24	0.19 ± 0.02	0.33 ± 0.05	0.39 ± 0.05
5.1	13	8	1.23 ± 0.18	1.51 ± 0.16	2.42 ± 0.38
5.2	26	16	0.66 ± 0.06	1.23 ± 0.20	2.05 ± 0.41
5.2	40	24	0.46 ± 0.08	1.02 ± 0.11	1.30 ± 0.27

Source: Phalen, R.F., *Inhalation Studies: Foundations and Techniques*, CRC Press, Boca Raton, FL, 1984. With permission.

SMALL ANIMAL EXPOSURE SYSTEMS

When using Flow-Through inhalation delivery, the snout of the exposed animals projects directly into the lumen of the system containing the exposure atmosphere. As a consequence, the respiration of each animal on the system will affect the atmosphere presented to other animals undergoing exposure. In exposure systems involving multiple levels of animals, the concentration of the test article will commonly be reduced at each animal level on the system. In

Directed-Flow exposure arrangements, there is a separate delivery of aerosol to each animal, and the exhaust from each animal passes directly to waste rather than becoming available to others on the exposure system. Consideration must be given to study design, test article, aerosol generation system type, dose levels, and airflows when choosing between a Flow-Through and a Directed-Flow exposure system as both snout-only exposure system types are appropriate for the delivery of test article atmospheres to animals (Figure 6.17; Table 6.61).

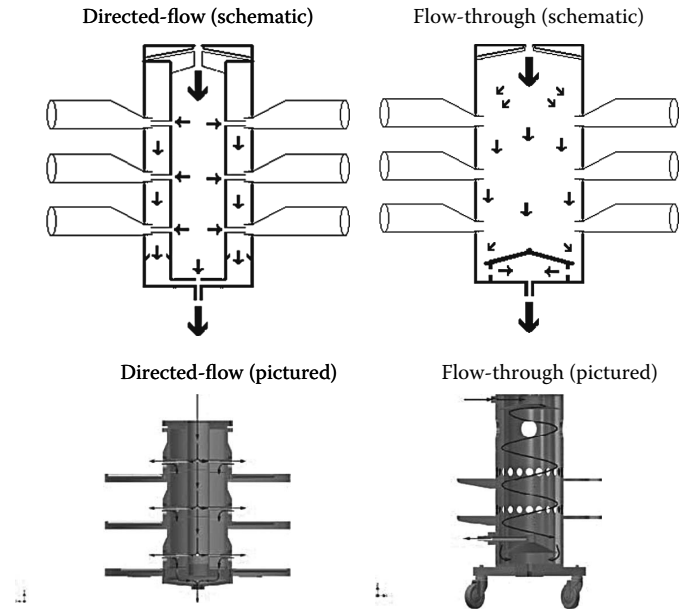


FIGURE 6.17 Directed-flow exposure and flow-through systems.

TABLE 6.61
Comparison of Directed-Flow versus Flow-Through Exposure Systems

Directed-Flow Exposure Systems		Flow-Through Exposure Systems	
Characteristic	Impact	Characteristic	Impact
Internally complex	Inlet and exhaust airflow balance is critical and must be monitored continuously to minimize contamination of the surrounding environment or systematic protection of animals from the intended exposure by a sheath of clean air.	Internally simple	Simple to operate and tolerant of variations in delivered airflows. Can be operated successfully at slight negative pressure (inward leakage of air from the surrounding environment) without adversely impacting the animal exposures.
	Each animal has its own aerosol delivery tube—the exposure zone is contained within the restraint tube.		No separate delivery tubes—the snout of the animal typically projects into the lumen of the exposure system.
	Can result in particle size stratification with smallest particle size at the top of the system and largest particle size at the lowest system levels.		Particle size stratification rarely seen as large aggregates and particle sediment passes directly through to the exhaust location.
	Generally less suitable for high concentrations of particulate due to changes in airflow associated with partial delivery tube occlusion by deposited powder.		A simple tube that is suitable for delivery of all achievable aerosol concentrations.
	For liquid aerosols, droplet sedimentation in the individual delivery tubes can provide an attractive source of fluid to confined and dehydrated animals, which then consume a significant additional oral dose.		No individual delivery tubes to consider.
	For particulate atmospheres, test article may sediment at the outlet of the individual delivery tubes and can be actively consumed by the animals to provide a significant oral dose—active consumption is particularly associated with test articles that have been diluted with a vehicle such as lactose.		Sedimented material is not accessible to the exposed animals.
	Can be labor-intensive to clean in routine use and difficult to decontaminate following periods of use.		Generally simple to clean and decontaminate.

(continued)

TABLE 6.61 (continued)
Comparison of Directed-Flow versus Flow-Through Exposure Systems

Directed-Flow Exposure Systems		Flow-Through Exposure Systems	
Characteristic	Impact	Characteristic	Impact
Each animal separately supplied with aerosol	Each animal receives its own atmosphere that is then drawn directly to waste. As a consequence, there is: No depletion of aerosol concentration; No O ₂ depletion; No supplementation of CO ₂ or humidity.	Animals are exposed to a common aerosol	The atmosphere delivered to each level of animals has been impacted by the preceding animal levels. As a consequence, there is: Reducing aerosol concentration with successive animal levels; O ₂ depletion; Supplementation of CO ₂ and humidity. Impact of stratification can be minimized by rotation of animals on system.
Employ ventilated restraint tubes	Tubes commonly low volume and ventilated with tail exposed to allow thermoregulation. Pressure and flow fluctuations are typically balanced by flow through the restraints. May systematically protect animals from aerosol or contaminate the surrounding containment environment with test article. Increased risk of restraint associated injuries with ventilated tube designs.	Typically employs unventilated and sealed restraint tubes	Large surface area provides thermoregulation heat exchange but less effective in this respect to ventilated and tail out restraints. Sealed tubes ensure pressure and volume-balancing airflow into a system does not take place through the restraints.
Small internal system volume and rapid equilibration	Associated with wide variations in momentary concentration if generation system fluctuations occur. System equilibration is typically measured in seconds and therefore good for reactive test articles. Short residence time can be beneficial for highly reactive test articles and materials that are subject to damage by desiccation when exposed to dry air. May be of insufficient volume to allow evaporation of droplets to an inhalable size May require the addition of large prechamber for use with Metered Dose Inhaler (MDI) canisters to increase residence time for propellant evaporation.	A relatively large internal system volume	Minimal risk of injury due to large volume design. High buffering capacity to dampen the influence of fluctuations in aerosol generation. Equilibration is measured in minutes. Extended residence time for aerosol in the system can negatively impact the integrity of test articles subject to desiccation damage.
Sensitive to airflow variations	Excessive inlet flow results in systematic and variable test article body burden for the test system and risks work environment contamination with concomitant risk of cross-contamination of control animals and technical personnel.	Operated at negative pressure	Large volume allows evaporation from liquid droplet atmospheres reducing particle size to inhalable size. Allows evaporation of vehicle materials for atmospheres generated from MDI canister formulations. Systems can be operated with an exhaust flow that exceeds the inlet flow substantially reducing the risk of test article escape into the surrounding containment and of cross-contamination of nearby control animals. The modular nature of the units provides opportunity for air to "leak" into the systems even though the restraint tubes are generally sealed.

TABLE 6.61 (continued)
Comparison of Directed-Flow versus Flow-Through Exposure Systems

Directed-Flow Exposure Systems		Flow-Through Exposure Systems	
Characteristic	Impact	Characteristic	Impact
	Excessive exhaust flow can systematically protect a proportion of the animals placed on the system.		Higher exhaust than inlet airflow acceptable.
	Sampling airflow has to be returned to the exposure system to maintain overall airflow balance.		Low flow rate sampling airflow rarely needs to be returned to the exposure system. Sample flows exceeding 10% of overall system flow should be returned to the base of the exposure system.
Can be operated at lower airflows relative to Flow-Through systems	Individual delivery of atmosphere allows airflows approaching the combined RMV of animals on the system.	Typically operated at airflows greater than similarly sized Directed-Flow systems	Airflow commonly calculated to ensure no greater than 15% aerosol concentration variation from the top exposure level to the bottom in order to ensure animal welfare standards and minimize delivered dose variations.
	System operated at low airflow per animal can significantly reduce the test article needs to complete a study relative to a Flow-Through type system.		Will consume more test article than a correspondingly scaled Directed-Flow system whenever higher airflows are employed.
Chamber design may limit number of animals dosed on the exposure system	Complex chamber design relative to the number of animal ports available for exposure may be a limiting factor in terms of number of animals (group size) that can be dosed simultaneously.	Chamber design will generally support a larger number of animals	Simple chamber design allows for large number of available animal ports per system level. Can accommodate large number of animals required per group for typical carcinogenicity study.

LARGE ANIMAL EXPOSURE SYSTEMS

For large animal species such as the dog, primate, and mini-pig, the most common delivery method is through the use of a face mask delivery system with or without an included airway or tongue depressor. During exposure, each animal is fitted with a mask that is secured to the animal's head with straps or through the use of a mask-covering muzzle. Each mask will typically incorporate a seal between the mask and the animal's face to prevent any leakage of test article during exposure. The test article aerosol may be distributed through individual flexible aerosol hose or delivered by rigid connections from a central expansion volume.

Aerosol exhaust from the masks is usually taken directly to waste to eliminate rebreathing of the aerosol (Directed Flow). An extract system is attached downstream of the face mask positions for both approaches and will commonly include a local filtration of aerosol waste.

A second method of inhalation administration to large animal species is via an oropharyngeal airway. The aerosol generation methods can be similar in design to those used for face mask exposure or based around an individual

generation device, commonly a clinical device or platform. Such systems will commonly use less test article than a corresponding mask delivery, but the duration of exposure is limited by the tolerance of the animal model. For exposure durations greater than 10 minutes, manpower costs can become prohibitively expensive for oropharyngeal delivery due to the necessity for individual handling. As with face mask exposures, where necessary due to pharmacology or total delivered dosage, multiple exposures can be conducted during each day. This is normally up to a practical maximum of 3 occasions per day.

Irrespective of the method of aerosol delivery selected, the test article must be administered in a manner that provides stable, reproducible exposure concentrations of an appropriate particle size for the test animals (generally accepted by US EPA, OECD, and US FDA to be 1–3 µm mass median aerodynamic diameter [MMAD] for small animals and 1–4 µm MMAD for large animals). Exposure concentration and particle size must be measured routinely to demonstrate that achieved values are within an acceptable range of target values, or to provide information needed to bring them within the desired ranges (Tables 6.62 and 6.63).

TABLE 6.62
Relative Advantages and Disadvantages of Face-Mask Exposure Systems

Face-Mask Exposure Systems		
Characteristic	Advantages	Disadvantages
Type of materials	Can be used for particulate, liquid, MDI, and gas/vapor delivery.	May not always be able to use clinical device without modification.
Airflow/pressure	Requires lower airflow than WBE, thus less test article usage. Inlet and extract airflows typically balanced.	Minimum airflow determined by respiratory requirements of animals on system. Buffering system needed to account for animal's impact on the system and ventilatory requirements. Imbalance in airflow may cause test article to leak from the system (positive) or animal distress (negative) due to lack of air.
Generation device typically attached to central expansion conditioning chamber fitted with variable number of aerosol delivery tubes	Allows expansion of aerosol, reduces the risk of the formation of aggregates and deposition of larger particles by sedimentation.	Excessive sedimentation in an aerosol conditioning chamber and delivery pipe network may result in low generation efficiency.
Aerosol delivery tubing and piping	Individual animal dosing with directed flow type delivery (no rebreathing). Wide variety of tubing materials available.	The length and diameter of connecting pipe network as well as internal tubing wall structure (corrugated vs. smooth) may impact delivery efficiency and aerosol concentration. Plastic tubing types may result in static charge accumulation in particulate aerosols. Consideration should be given to the potential for leachates and extractables from plastic tubing when delivering liquid aerosols.
Face mask with or without included airway or tongue depressor	Individual mask per animal. Seal between mask and muzzle limits leaks. Test article delivery confined to snout/muzzle.	A significant proportion of oral and nasal turbinate delivery will occur but can be limited by the use of a tongue depressor tube. Dead space in mask may reduce exposure and result in respiratory changes if sized incorrectly. If not properly designed and fitted, it may cause pressure lesions on snout area.
Animal restraint may include slings, chairs, and stocks, or can involve trained compliance	Restraint permits delivery of the test atmosphere for the required exposure duration.	Respiratory parameters may be negatively affected by some forms of restraint.
Exposure duration	As few as 2 min but typically 10 min up to 4 h.	Time-consuming relative to other routes of administration.

TABLE 6.63
Relative Advantages and Disadvantages of the Oropharyngeal (OP) Exposure Systems

Oropharyngeal Exposure System		
Characteristic	Advantages	Disadvantages
Can be used for particulate, liquid, and clinical platform deliveries	Multipurpose. High doses achievable. Portable and self-contained exposure systems can be designed. Mimics clinical delivery methods. Test article can be continuously delivered or timed to coincide with the breathing pattern.	Individual dosing can be time-consuming and labor-intensive, resulting in higher study costs. If test article generation is not timed to coincide with the breathing pattern, animals may not receive intended exposure.
Airflow/pressure	Requires lower airflow than WBE and will typically require lower airflow than a corresponding face mask exposure. Reduced test article usage. May involve only tidal flow of air from the room environment. Inlet and extract airflows typically balanced for dynamic systems.	Animals may modify breathing to limit exposure if pharmacological effects are unpleasant. Buffering system needed to account for animal's impact on the system and ventilatory requirements. Imbalance in dynamic airflow may cause test article to leak from the system (positive pressure) or animal distress (negative pressure) due to lack of air.

TABLE 6.63 (continued)
Relative Advantages and Disadvantages of the Oropharyngeal (OP) Exposure Systems

Oropharyngeal Exposure System		
Characteristic	Advantages	Disadvantages
In dynamic aerosol delivery systems, the generation device is commonly attached to central expansion conditioning chamber fitted with variable number of aerosol outlets with connected oropharyngeal tubes	An expansion chamber allows expansion of aerosol and reduces the risk of the formation of aggregates and allows deposition of larger particles by sedimentation.	Long retention times for test article aerosol in chamber can permit excessive sedimentation and result in reduced efficiency.
Aerosol delivery tubing and piping	Individual animal dosing with directed flow type delivery (no rebreathing). Wide variety of commercially available clinical aerosol tubing materials available.	Length, diameter, and structure of tubing and piping may impact efficiency and aerosol concentration. Plastic tubing may induce static charge on particulate aerosols. Consideration should be given to the potential for leachates and extractables from plastic tubing when delivering liquid aerosols.
Oropharyngeal tube	Tubes should be individually tailored to each study animal. Test article delivery directly to oropharynx without causing distress. Reduced risk of aerosol leakage with skilled operators.	Selection of an incorrect tube type, tube length, or diameter may cause injury to mouth/throat and result in reduced test article delivery efficiency. If incorrectly handled, aerosol can leak into the work environment or fresh air delivered around the outside of the airway.
Animal restraint may include slings, chairs, stocks, or manual methods	Restraint is essential to ensure animals receive test atmosphere for the required exposure duration.	Respiratory movement and respiratory parameters may be affected by some restraint methods.
Exposure duration	Typically short duration up to a maximum of 10 min in chronic toxicity studies.	Limited exposure duration to a maximum of 15 min in short-duration dose range-finding studies. May require multiple daily exposure sessions to achieve target dose resulting in higher study cost.

ATMOSPHERE GENERATION METHODS

Administration of test articles in the gaseous/vapor state occurs for those chemicals that are normally a gas at room temperature or are solids or liquids with a vapor pressure high enough to achieve the target exposure concentration. The former are referred to as gases, while the latter are called vapors. Administration of test articles as gases is typically accomplished using flow dilution techniques. Administration of vapors is typically accomplished using gas scrubbing or evaporation–volatilization techniques. Because of the complexity and diversity of these systems and their unique application to specific studies, they cannot all be described here.

Administration of test articles as aerosols is performed for chemicals that are normally liquids or solids at room temperature. These test articles do not possess a vapor pressure high enough to allow generation of the desired target concentration in the gaseous/vapor state. Aerosols originating from liquids can further be classified as liquid droplet aerosols, while those originating from solids can be classified as dust or particulate atmospheres.

Administration of liquid droplet aerosols is typically accomplished using spray atomization, nebulization, or evaporation–condensation techniques.

Administration of dusts is typically accomplished using either a nonsegregating technique such as a dust feed or a segregating technique such as a fluidized bed or sonic generator.

Because of the complexity and diversity of these systems and their unique application to specific studies, they cannot all be described here.

Fluid for vapor generation systems may be delivered as a simple bolus directly into the generator, by laboratory pump (commonly valveless piston or peristaltic) or from a syringe held in a syringe pump. Components should routinely be checked for compatibility with the material to be generated in advance of use. For vapor atmospheres, where it is not appropriate to have measurable aerosols, it is important to confirm the absence of condensate. This can be achieved by light scattering or laser diffraction techniques.

With the exception of gas and vapor atmospheres, irrespective of the test article type and method of aerosol generation, it is commonly necessary to control the charge characteristics or particle/droplet size of aerosol delivered to the exposure system. For particulate atmospheres, this will generally involve either the removal or comminution of large particles/aggregates in order to achieve a particle size that will be inhalable and/or the reduction of electrostatic charge induced during aerosol generation. For liquid droplet atmospheres, the reduction of particle size is rarely necessary, but maintenance of the particle size may be required to keep the atmosphere in the ideal range for the species receiving the exposure. (Tables 6.64 through 6.67; Figures 6.18 through 6.26; Tables 6.68 through 6.70).

TABLE 6.64
Commonly Used Generation Systems for Particulate Test Articles

Generator Type	Commercially Available Examples	Characteristics
Scraper-type mechanisms	Wright Dust Feed by multiple manufacturers, TSE Budiman Generator and Auger systems.	Particulates that can be compacted into a solid pellet but readily de-agglomerated to their original particle size.
Brush mechanisms	Rotating Brush Generator by CR Equipements (SA and Palas GmbH).	Particulates that can be compacted into a loose column and pushed by piston or conveyor belt into the path of a moving brush. Particularly effective for highly structured particulates that would be damaged by compression necessary for scraper-type delivery methods.
Fluidized powder generators	Thermo Systems Inc. and Palas GmbH.	The fluidized movement of beads in an airstream is used to disrupt aggregates in powder presented by a conveyor system. Large particles remain in the generator due to sedimentation.
Capsule and cell based generator systems	Not commercially available.	Generate atmospheres from loose bulk powder that is aliquoted into capsules. Achieved concentration determined by a combination of capsule usage rate, capsule fill, and airflow.
Turntable generators	Palas, Sibata, TSI, and TSE.	Powder dispensed into a groove or precision pockets in a delivery platter and generated by pickup and ejection by an air mover.
Vilnius generator	EMMS and CH Technologies.	Loose powder dispensed into a small reservoir containing a turbine. The rotating turbine and agitation from the membrane at the base of the reservoir work in unison.

TABLE 6.65
Commonly Used Generation Systems for Liquid Test Articles

Generator Type	Example Manufacturers	Characteristics
Compressed air nebulizers	Pari, Cardinal Health, Omron, DeVilbiss, Graham-Field, Invacare, Mabis DMI, Philips Respironics Teleflex-Hudson RCI, Allied Healthcare, BGI, Medic-Aid and CH Technologies	Needs a separate source of compressed air, high shear used to disrupt a column of liquid, temperature drops during aerosol production due to evaporation of vehicle. May damage structured/energy-sensitive test articles. Will increase the concentration of solutes due to evaporation and reflux. Typically produces aerosols within an appropriate respirable range.
Mesh micropump nebulizers	Omron, Aerogen, Pari, Philips Respironics and Beurer	Direct droplet formation through a micromesh screen using energy from a piezoelectric element. No heating of test article, silent operation, low waste of test article, no air involved in generation process allowing high local concentrations. Reservoir contents are not refluxed; therefore, no concentration effect on contents. Mesh screens may progressively block during periods of use reducing the rate of test article delivery.
Ultrasonic nebulizers	DeVilbiss, Omron and Beurer	Uses a focused high-frequency wave that causes vibration in a piezo element, very high output possible, no compressor, high (relatively) waste and unsuitable for heat-labile materials, almost silent during operation.

TABLE 6.66
Commonly Used Generation Systems for Gas and Vapor Test Articles

Generator Type	Commercially Available Examples	Characteristics
Glass vaporizers	Custom glass manufacturers made to user specifications.	Fluid for vaporization dispensed onto a glass sinter or into a simple glass vessel with diluent air blown through or over the liquid. Vessel or air may be heated to aid volatilization.
Gas Flowmeter and control valve dispensing J tube	Any precision flowmeter (variable area, mass flow), needle valves, and pressure regulators. Custom glass manufacturers made to user specifications	Pure gas or vapor delivered into a diluent airflow and then directed to the exposure system. Liquid for vaporization dispensed into a J tube containing an inert media (e.g., glass beads) through which the diluent gas flow is metered. The system is commonly heated to aid volatilization.
Countercurrent generator	Custom glass manufacturers made to user specifications.	Similar to J tube but generally larger scale. Will commonly include a heating element, heated jacket, or heated counterflow of water or oil to aid volatilization. Fluid for generation will generally be dispensed onto a large surface area and have a relatively low surface flow rate.
Vapor delivery from a low-pressure cylinder	Any precision flowmeter (variable area, mass flow), needle valves, and pressure regulators.	Care should be taken to ensure that vapor delivery is maintained, commonly achieved by heating the cylinder body. Systems can suffer from condensate formation in pipes and flowmeters, resulting in intermittent delivery and poor concentration control.
Liquid delivery from a low-pressure cylinder	Any precision flowmeter (variable area, mass flow), needle valves, and flow regulators connected to a bottom feed valve.	Care must be taken to ensure the head pressure is maintained. Liquid is commonly dispensed to one of the generation systems described earlier.
Gas delivery from a high-pressure cylinder	Dual stage gas regulator with appropriate connection types and gauges together with precision flowmeter (variable area, mass flow), needle valves, and flow regulators.	Simple dilution with the vehicle (typically compressed air). Mixing of the gas and vehicle is essential for high- or low- density gases.

TABLE 6.67
Measures Used to Control Particle Size and Aerosol Charge

Controlling Measure	Commercially Available Examples	Characteristics
Radioactive charge neutralizers	TSI and CR Equipment (blank neutralizer tube only).	Generally most effective at low flow, requires significant operator training and radiation monitoring. Encapsulated sources such as Kr-85 and Po-210 are safer than naked radionuclides such as Ni-63.
Addition of humidity	User produced from standard commercial items or custom-produced glassware.	Introduction of humidity into some aerosols may be unacceptable, but in many instances will significantly reduce charge at low cost. Humidity may be used to increase or maintain particle size for some liquid aerosols if the MMAD is too small.
Corona discharge charge neutralization	Simpco, Topas, Cole Static Control, Ground Zero Electrostatics, and TAKK Industries Inc.	May cause particle production due to sputtering, and the resulting ozone production and delivery to the exposure system may influence study outcome. Will operate at high airflows but only suitable for clean air diluent flows.
Elutriation (deliberate reduction in air velocity)	Custom glass and plastics and metal work manufacturers made to user specifications and CR equipment	Large particles sediment against airflow. Inexpensive passive systems.
Cyclones	BGI and Laboratory Glassware suppliers.	Passive device in the delivery line, performance dependent on airflow rate and may not work efficiently at low flows. May impact achievable aerosol concentration and delivery efficiency due to filtering effect of device.
Distance (sedimentation)	Any smooth bore plastic, glass, or metal tubing and laboratory items readily available.	Inexpensive, may induce charge in the delivered aerosol, similar to elutriation but without planned reduction in aerosol velocity.

(continued)

TABLE 6.67 (continued)
Measures Used to Control Particle Size and Aerosol Charge

Controlling Measure	Commercially Available Examples	Characteristics
Turbulence	Any corrugated bore tubing.	Increases large particle/droplet sedimentation. Flow-dependent and may induce static charge.
Jet milling (fluid energy mills)	Food and Pharma Systems Srl, Sturtevant Inc., Hosokawa Micron Group, Jet Pulverizer Co., Glen Mills Inc., Fluid Energy Processing and Equipment Co.	Produce a narrow range of particle size without significant heating of product. Can be performed dynamically as aerosol is generated minimizing product contamination and cross-contamination risks. Systems are passive, and minimal maintenance for pharmaceutical products but may be damaged by abrasive materials. Airflow demands for the milling process can substantially increase compound consumption for low-volume exposure systems.
Jar milling/ball milling/cryogenic ball milling/planetary ball milling	US Stoneware Inc., Glen Mills Inc., Union Process Inc. Retsch GmbH. and Laarmann Group B.V.	Motorized version using grinding medium contained in grinding jar to reduce particle size. Milling time typically 30 min but alternate durations can be used. Product typically requires sieving prior to use for inhalation delivery. Some test article loss due to adhesion to grinding medium and jar walls. Material lost from ceramic milling beads may contaminate abrasive test articles. Cryogenic milling can protect product, reduce processing time, and provide a smaller final product particle size.
Mortar and pestle	Laboratory equipment suppliers	Manual operation makes consistency of product variable. Motorized versions provide a more consistent final product. Particle size can be highly variable, and product may require sieving prior to use for inhalation delivery.
Mechanical Mills (ultracentrifugal, cross beater, hammer, disk, and others)	Multiple mill types from a broad range of manufacturers.	High-energy mills that will commonly heat the product during processing. Care must be taken ensure heat-labile materials are not damaged by the process. Some mill types may be tolerant of cryogenic operation employing the co-milling of dry ice pellets.
Test sieves	Laboratory equipment suppliers.	Manual or mechanical removal of large particles and retention of the size range desired for aerosol generation.

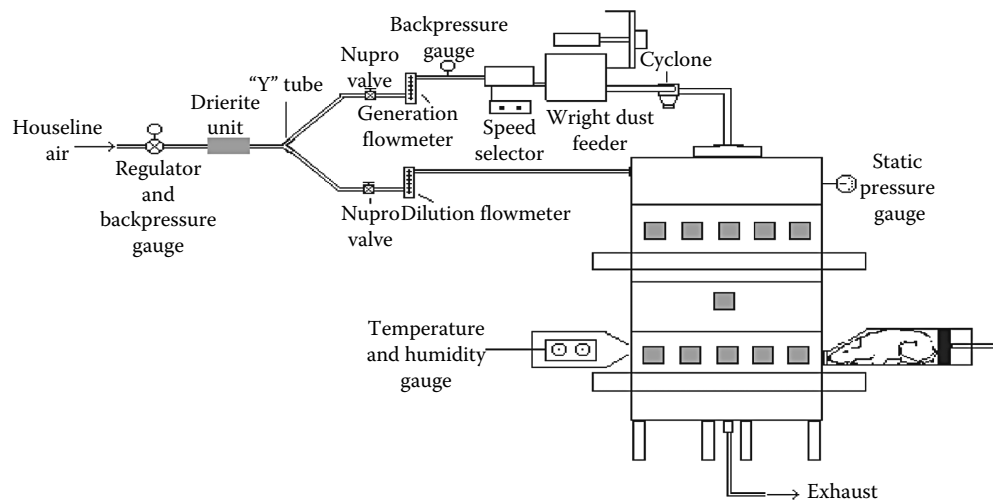


FIGURE 6.18 System for generation of a dust atmosphere into a nose-only exposure system. A particulate generator (Wright dust feed is shown) is used to disperse the aerosolized powder through a cyclone or elutriator, for removal of large particles, into the nose-only exposure chamber from which the test animals breathe. In-line jet milling can be added between the dust generator outlet and the exposure system to comminute the aerosol output to the exposed animals.

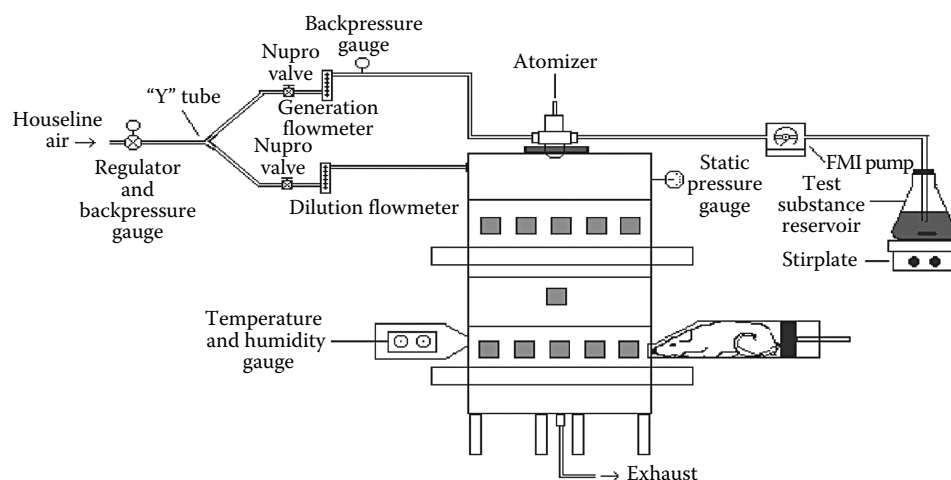


FIGURE 6.19 System for generation of a liquid aerosol atmosphere into a nose-only exposure system. A spray atomizer, compressed air, vibrating mesh, or ultrasonic nebulization system is used to disperse the aerosolized liquid, fed from a reservoir with a fluid metering pump or syringe pump, into the nose-only exposure chamber from which the test animals breathe. A cyclone or elutriator can be used to remove large droplets from the air entering the exposure system or the droplet size reduced by evaporation by the addition of dry diluent air.

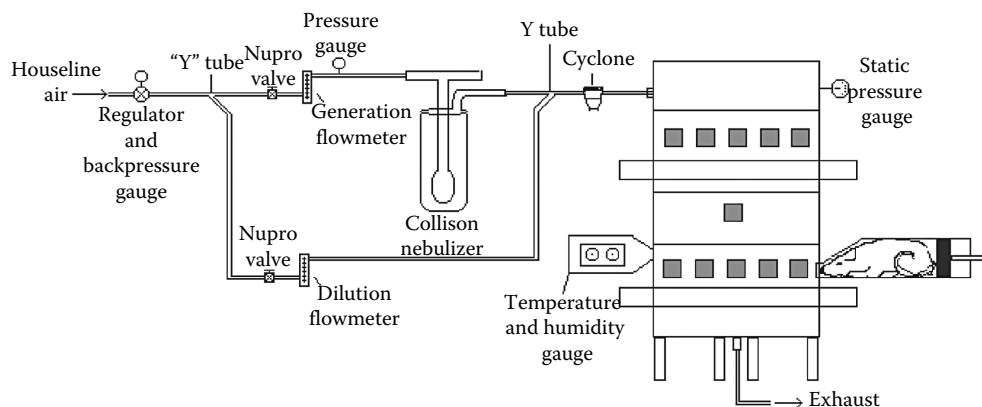


FIGURE 6.20 System for generation of a liquid aerosol atmosphere into a nose-only exposure system. A collision nebulizer is used to disperse the aerosolized liquid through a cyclone for removal of large particles into the nose-only exposure chamber from which the test animals breathe.

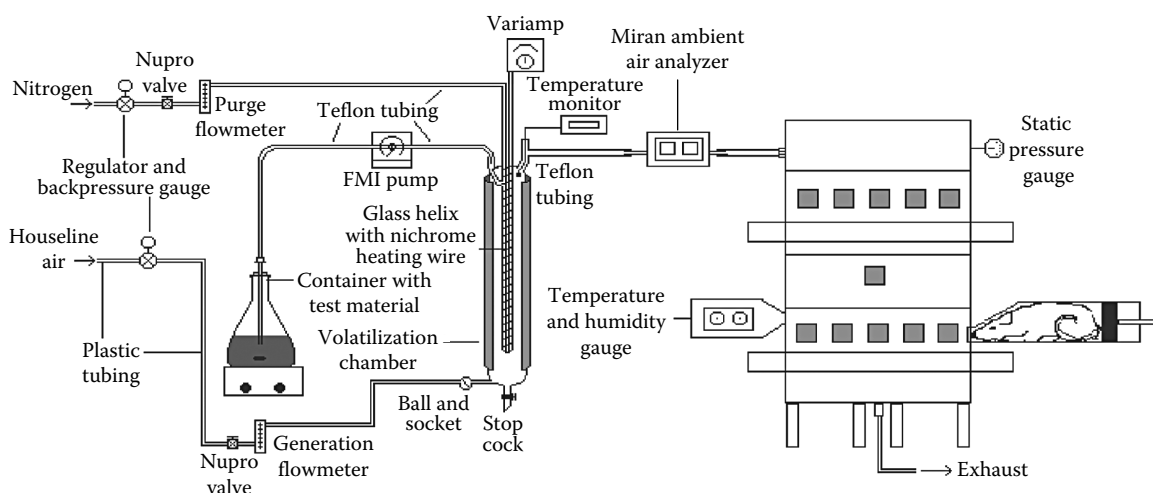


FIGURE 6.21 System for generation of a vapor atmosphere into a nose-only exposure system. A countercurrent volatilization chamber (heated with a nichrome wire or hot water jacket, if needed) is used to disperse the vaporized liquid, fed from a reservoir with a fluid metering pump, into the nose-only exposure chamber from which the test animals breathe.

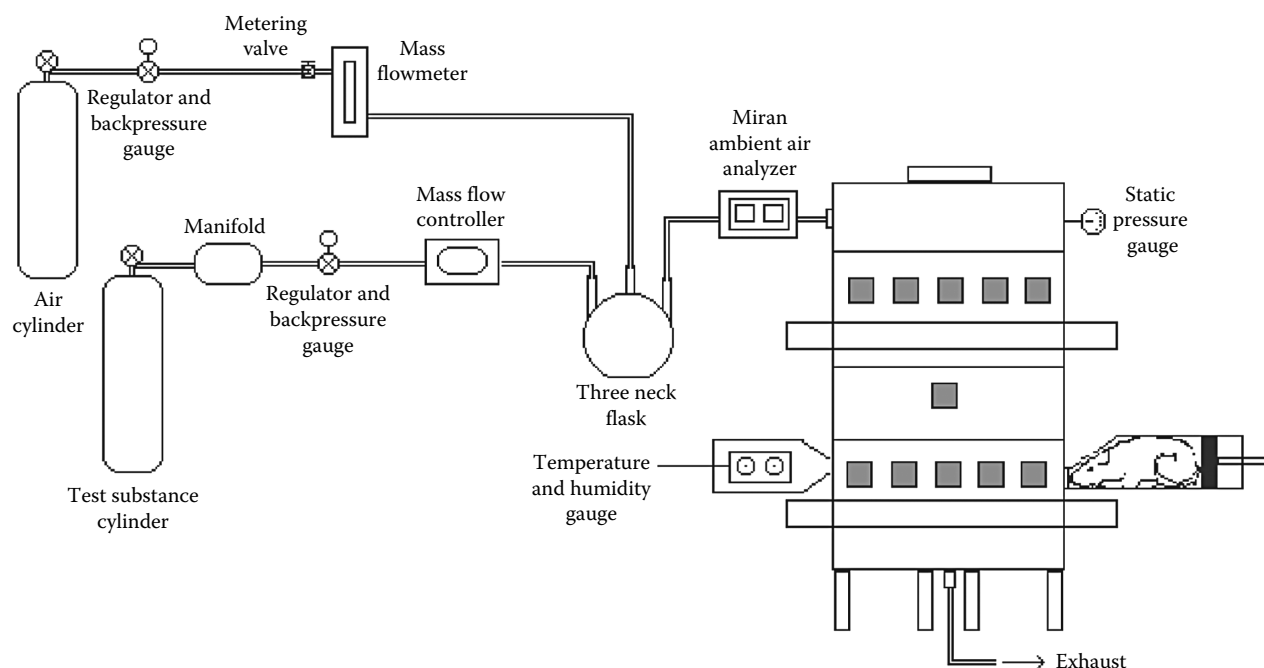


FIGURE 6.22 System for generation of a gas atmosphere into a nose-only exposure system. A mass flow controller is used to disperse the gas, fed from a pressurized cylinder, into the nose-only exposure chamber from which the test animals breathe.

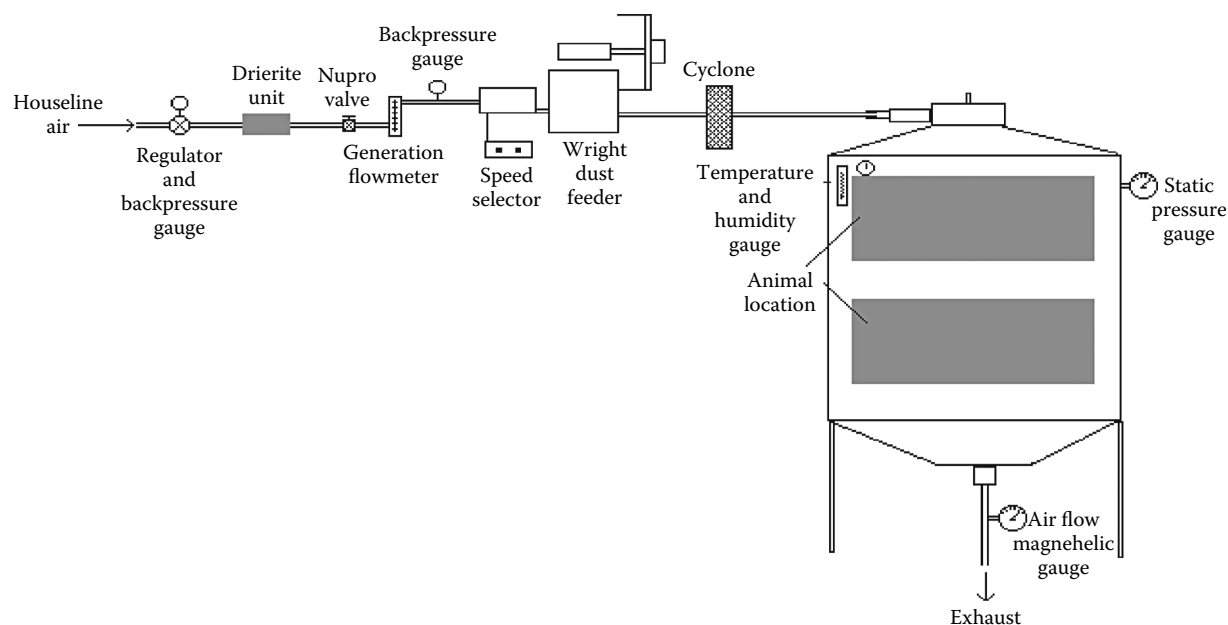


FIGURE 6.23 System for generation of a dust atmosphere into a 1000-l whole-body exposure chamber. A dust generator (Wright dust feed shown) is used to disperse the powder through a cyclone, for removal of large particles and aggregates, into the whole-body exposure chamber within which the test animals are placed.

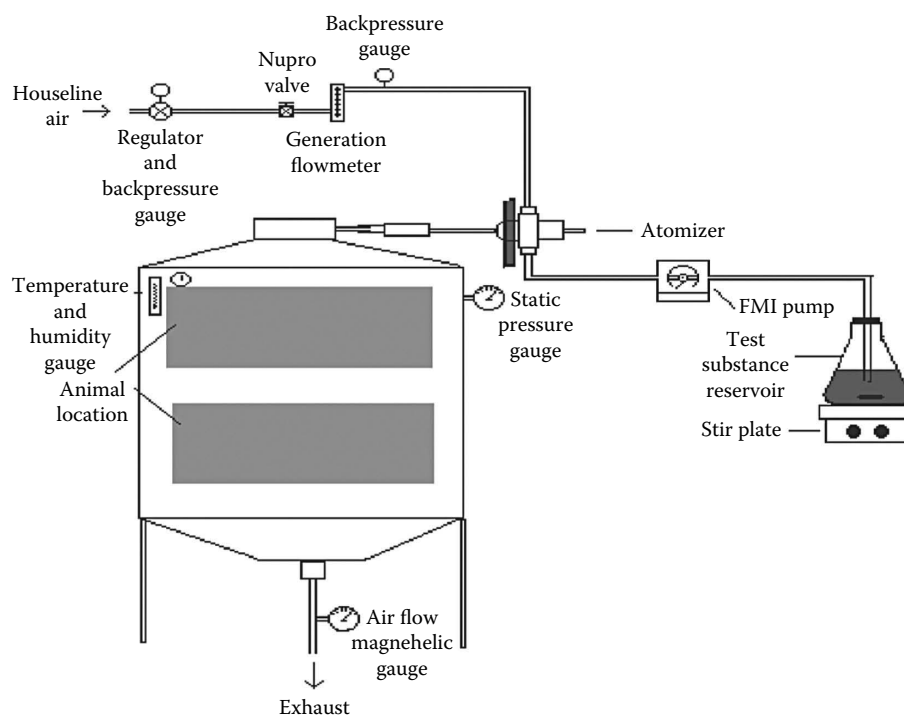


FIGURE 6.24 System for generation of a liquid aerosol atmosphere into a 1000-l whole-body exposure chamber. A spray atomizer is used to disperse the aerosolized liquid, fed from a reservoir with a fluid metering pump, into the whole-body exposure chamber within which the test animals breathe.

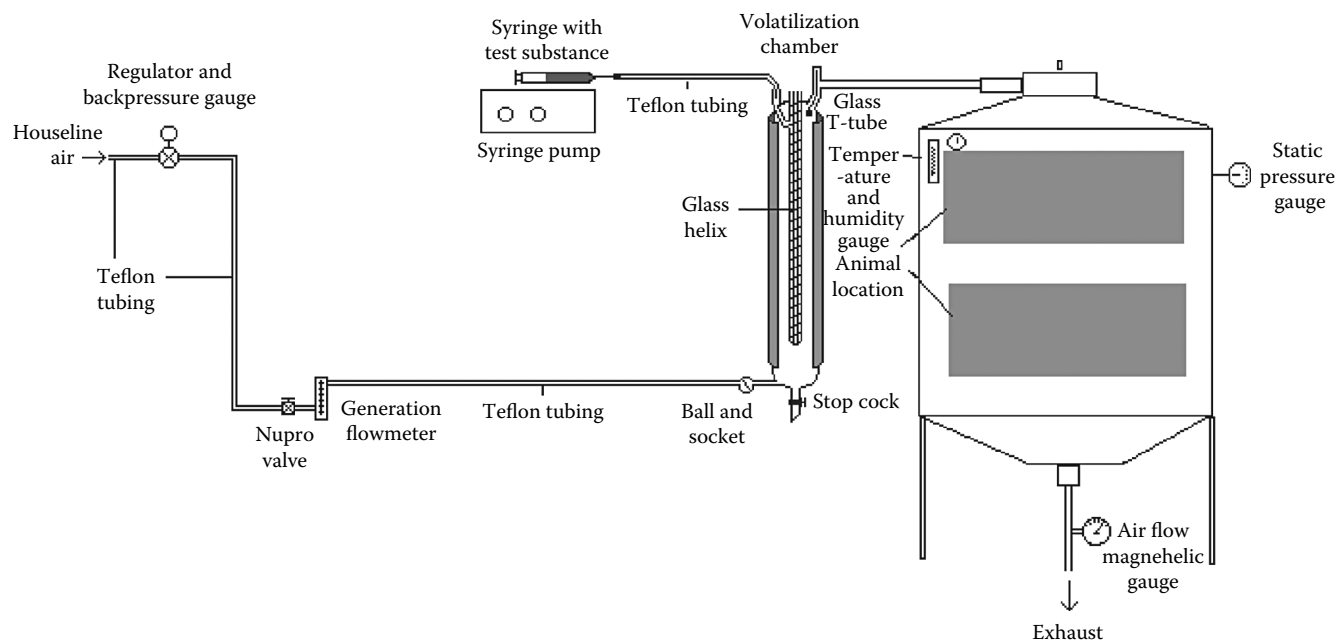


FIGURE 6.25 System for generation of a vapor atmosphere into a 1000-l whole-body exposure chamber. A countercurrent volatilization chamber (heated with a nichrome wire, if needed) is used to disperse the vaporized liquid, fed from a syringe pump, into the whole-body exposure chamber within which the test animals breathe.

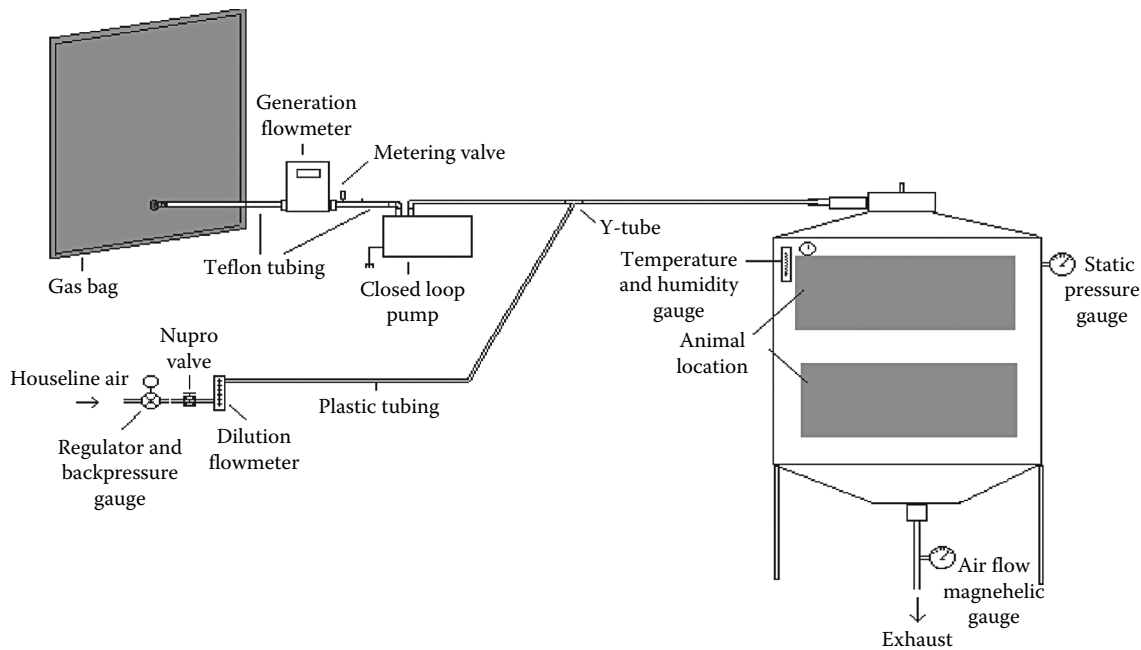


FIGURE 6.26 System for generation of a gas atmosphere into a 1000-l whole-body exposure chamber. A flowmeter is used to disperse the gas, fed from a gas bag with a vacuum pump, into the whole-body exposure chamber within which the test animals breathe.

TABLE 6.68
Characteristics of Nebulizers

Nebulizer	Operating Pressure (psi)	Flow Rate (L/min)	Output Concentrations (µg/L)	MMAD (µm)	GSD ^a
Laskin	20	84.0	4.8	0.7	2.1
In Tox	30	25.0	32.0	6.1	1.9
Solosphere	20		1.5	4.5	
Ohio	20		0.5	4.5	
DeVilbiss	20	16.0	14.0	3.2	1.8
Hospitak	20	11.0	23.0	1.0	2.1
Collision	20	7.1	7.7	2.0	2.0
Retec X-70 N	20	5.4	53.0	5.7	1.8
Lovelace	20	1.5	40.0	5.8	1.8

Source: Reproduced from Moss, O.R. and Cheng, Y.-S., *Concepts in Inhalation Toxicology*, Hemisphere Publishing, Taylor & Francis, Washington, DC, p. 103, 1989. With permission. All rights reserved.

^a GSD, geometric standard deviation.

TABLE 6.69
Operating Characteristics of Compressed Air Nebulizers

Nebulizer	Applied Pressure (lb/in. ²)	Air Flow (L/min)	Aerosol Out (μL/L air)	Water Vapor Out (μL/L air)	Volume Median Drop Diameter (μ)	GSD
Dautrebande with open vent	5	11.2	1.0	9.7	—	
	10	14.9	1.4	9.6	1.7	
	20	21.2	2.3	8.6	1.4	1.6–1.7
	30	27.3	2.4	8.2	1.3	
3-Jet Collison	15	6.1	8.7	12.6		
	20	7.1	9.0	14.8		
	30	9.4	9.0	19.4	About 2	About 2
	40	11.4	9.3	23.5		
	50	13.6	10.4	27.9		
De Vilbiss® No. 40 with closed vent	15	12.4	15.5	8.6	4.2	
	20	16.0	14.0	7.0	3.2	1.8–1.9
	30	20.9	12.1	7.2	2.8	
Lovelace	20	1.34	(34) ^a	12	6.9	1.7
	30	1.81	(22) ^a	11	4.7	1.9
	40	2.28	(15) ^a	9	3.1	2.2
	50	2.64	(19) ^a	11	2.6	2.3
(Nebulizer chilled to 0°C)	20	1.34	55	1	—	—

Source: Phalen, R.F., *Inhalation Studies: Foundations and Techniques*, CRC Press, Boca Raton, FL, 1984. With permission.

Note: GSD, Geometric standard deviation.

^a Calculated.

TABLE 6.70
Characteristics of Dry Powder Generators

Generator	Delivery Mechanism	Dispersion Mechanism	Flow Rate (L/min)	Mass Concentration (mg/m ³)	Test Material
NBS	Gravity	Venturi	50–85	1500 and larger	Nonsticky powder
Wright Dust Feed	Rotating blade	Airstream	10–40	2–1100	Compactable powder
TSI model 3410	Rotating brush	Airstream	10–50	1–100	Nonsticky powder
MDA Micro Feed	Rotating disk	Venturi	30–50	10–300	Nonsticky powder
TSI model 3433	Rotating disk	Venturi	12–21	0.3–40	Nonsticky powder
Lovelace 4-in. FBG	Gravity	Fluidized bed	200	1–100	Nonsticky powder, fiber
TSI model 3400	Chain conveyor	Fluidized bed	5–15	10–100	Nonsticky powder
Jet-O-Mizer	Screw feed	Air mill	300–400	2–1000	Sticky powder
Battle Micronizer	Dual brush	Air mill	30–50	5–5000	Sticky powder
Microjet	Screw feed	Air mill	300–1000	2–1000	Fiber

Source: Reproduced from Moss, O.R. and Cheng, Y.-S., *Concepts in Inhalation Toxicology*, Hemisphere Publishing, Taylor & Francis, Washington, DC, p. 103, 1989. With permission. All rights reserved.

INHALATION EXPOSURE ENVIRONMENT

The inhalation exposure laboratory environment, although not strictly a component of an exposure system, can have a profound impact on the delivery of some test articles.

Many particulate and liquid droplet aerosols will accumulate a significant electrostatic charge during aerosol production and during subsequent travel from the aerosol generator to the animal exposure zone. This charge can be exaggerated or minimized based on the environmental conditions surrounding the exposure system. In a typical toxicology laboratory environment, humidity will be controlled in the range of 30%–70% RH; however, at the lower end of this range, a significantly greater charge may be accumulated by the aerosol. The acquisition of an electrical charge on an aerosol is commonly associated with losses onto the walls of the exposure system. Such losses may be acceptable in terms of the efficiency of delivery but should remain constant from one occasion of generation to the next in order to provide an acceptably constant delivered dosage to the animal model. This requires either maintenance of a higher degree of control over the environmental conditions or the use of a charge elimination/reduction instrument as indicated earlier. In an ideal arrangement, the charge on the aerosol would be dispersed to a value close to Boltzmann equilibrium (no net charge) to encourage the optimal delivery efficiency. In practice, even when charge reduction methods are employed, the particle number concentration in toxicology studies will commonly exceed the capacity of the neutralizing method, and some charge will be retained or re-acquired after treatment.

In vapor exposures involving concentrations that approach the maximum concentration that can be maintained, both the surrounding environmental temperature and the exposure system internal humidity can be critical to maintaining the delivered atmosphere in the vapor phase. A drop in room temperature, particularly in combination with increased exposure atmosphere humidity due to test model respiration, may result in the presence of test article droplets. When calculating the maximum vapor concentration that can be achieved, allowance must be made for the impact of these variables.

SAMPLING

All inhalation toxicology exposure atmospheres must be evaluated at intervals to demonstrate that the target exposure concentrations (and an appropriate particle size) and/or

dosages are delivered. These data are also necessary to permit informed decisions regarding adjustments to the exposure conditions.

During industrial or agricultural chemical studies, where exposures are typically up to 6 h duration, samples are commonly collected at approximately 60 min intervals during single exposure inhalation studies and every 1.5 h during repeat exposure inhalation studies.

Gas and vapor exposure concentrations are commonly determined using an infrared (IR) spectrophotometer or gas chromatographic (GC) methods. The IR is simpler to calibrate but only provides a total hydrocarbon concentration and does not quantify individual components in a complex mixture. The GC is more complex to calibrate but may be suitable to quantify individual components in a complex mixture. In either case, the instrument would be directly calibrated using standards of the test article. Samples for GC analysis may be drawn directly from the exposure systems to a chromatograph, collected directly in the gas or vapor phase into a gas bulb or gas tight syringe for injection on column, absorbed into a volatile liquid media for GC injection, absorbed onto a solid media for thermal desorption or absorbed onto a solid media for subsequent solvent extraction. Samples for IR analysis will typically be drawn directly into the instrument.

Aerosol exposures are commonly measured using gravimetric (weight difference) determination of test article collected onto a fiber or membrane filter or by direct trapping in a solvent for subsequent liquid or GC determination. Test article collected on filters may also be extracted and quantified with a gas or liquid chromatographic method.

Aerosol exposures also require the evaluation of the particle size to confirm the respirability of the atmosphere for the test animal species receiving exposure. Particle sizing is typically accomplished using cascade impactor techniques (with gravimetric and/or analytical determination of the collected sample) or using photometric techniques such as laser–light interception and time of flight. Particle sizing results are usually reported in terms of the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). The methodology for evaluation of cascade impactor samples is nicely discussed by the US EPA (<http://www.epa.gov/apti/bces/module3/distribu/distribu.htm>). The real-time measurement of particle size can also be used to confirm the absence of condensation droplets in atmospheres intended for delivery wholly in the vapor phase. (Table 6.71; Figures 6.27 through 6.35).

TABLE 6.71
Sampling Methodologies

Sampling Method	Commercially Available Examples	General Characteristics
Open face filtration—gravimetric analysis	Filter holders: BGI, TSE, and Cassella Filter media: Whatman, Pall, Millipore, etc.	Glass and Quartz Fiber—good general purpose depth filter media but may not trap aerosols below 0.5 μm diameter. Fiber loss onto filter holders and during handling may impact gravimetrically measured concentrations. Effective for particulate and nonvolatile liquid droplet atmospheres.
Open face filtration—extraction and chromatographic analysis		Glass and Quartz Fiber—good general purpose depth filter media may not trap aerosols below 0.5 μm diameter. Poor recovery from glass for some biologics. Binder-free media needed if extraction for analysis required. Membrane filters—many have limited trapping capacity before blockage. Small pore or mesh sizes may be excessively resistant to sample airflow. Hydrophobic filters may be associated with beading and loss of liquid droplet samples.
Bubblers and impingers and gas absorption traps	Custom manufacturers, VWR, and Ace Glass	Dilution of sample by trapping solvent may require extended sample intervals. Trapping efficiency has to be confirmed and may indicate requirement for multiple samplers in series. Loss of biologics onto glass or plastic surfaces. Occasionally poor recovery due to surface area. Metal bubblers less prone to surface losses, but operation is not visible to operators. Slow sampling rate often required to minimize carryover and optimize trapping efficiency.
Solid media absorption	Custom manufacturers, SKC, and Supelco	Thermal desorption directly onto GC possible but typically takes place into solvent. Desorption efficiency can be an issue. Extra handling can be associated with losses. Slow sampling rate typically needed to optimize trapping efficiency.
Cascade impaction	In-Tox Products, Thermo Fisher, MSP Corp, TSE Systems	Widely varying flow rates between devices. Low flow units more suitable in many testing situations. High flow units often acceptable for WBE chambers. Internal losses can be high. Can be used for many liquids, provided stage loading is not excessive. Can be prone to particle bounce.
Multistage liquid cascade impingers	May Multistage Impingers (specialist glassware manufacturers), Copley Scientific (MSLI)	High flow rates required may exceed available flow from an exposure system. Extremely low internal losses with careful sample handling. Some designs have a limited number of stages preventing the calculation of MMAD. Sample collection directly into solvent eliminates particle bounce.
GC analysis	Analytical equipment suppliers	Typically used for gas/vapor sample analysis. Online use requires GLP compliant software for data capture. May require extensive network of sampling pipes. Care must be taken to ensure equilibration of atmosphere within the sample lines and avoidance of condensate formation in the line. Off-line analysis requires appropriate trapping media or containers. Care must be taken to avoid loss of atmosphere sample from gas-tight syringe, gas bag, or other trapping media during transport to GC. Ensure appropriate consideration given to selecting seals/gaskets on sampling devices in relation to components in the test article (solvents, etc.).
IR analysis	Analytical equipment suppliers	Typically used for gas/vapor sample analysis. Online use requires GLP compliant software for data capture. May require extensive network of sampling pipes. Care must be taken to ensure equilibration of atmosphere within the sample lines and instrument cell and avoidance of condensate formation in the line or on the mirrors. Care must also be taken to ensure test article will not damage mirror coating and that mirrors are in good condition or clean after exposure to test atmosphere as this would impact vapor analysis.
Liquid chromatographic analysis (HPLC, UPLC)	Analytical equipment suppliers	Off line systems requiring the initial collection of filter or bubbler samples. The most commonly employed analytical systems employed for the quantification of inhalation exposure concentrations and particle size.

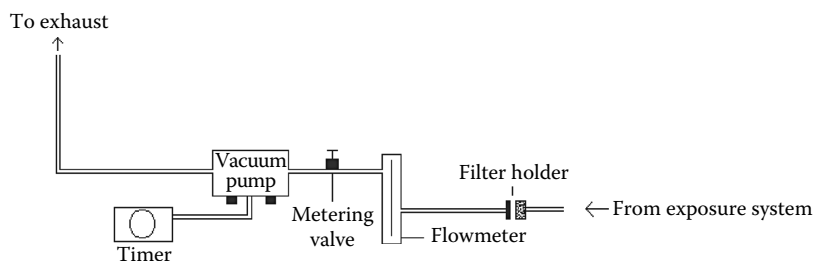


FIGURE 6.27 System for collection of filter paper samples of test atmospheres for gravimetric or analytical evaluation. A vacuum pump is used to draw the air sample through a glass fiber filter paper from the exposure system.

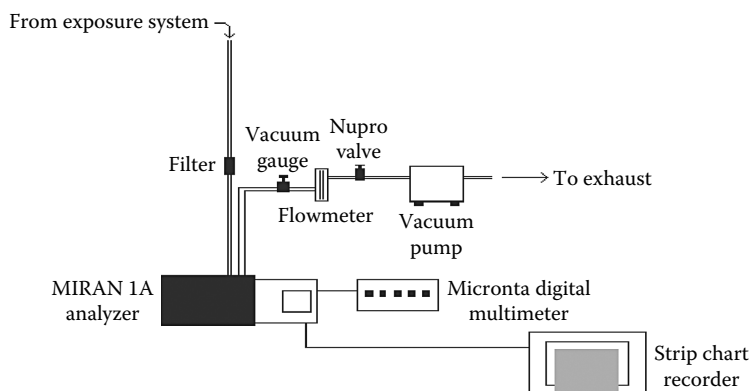


FIGURE 6.28 System for collection of air samples of test atmospheres for IR spectrophotometric evaluation. A vacuum pump is used to draw the air sample through a calibrated IR spectrophotometer from the exposure system.

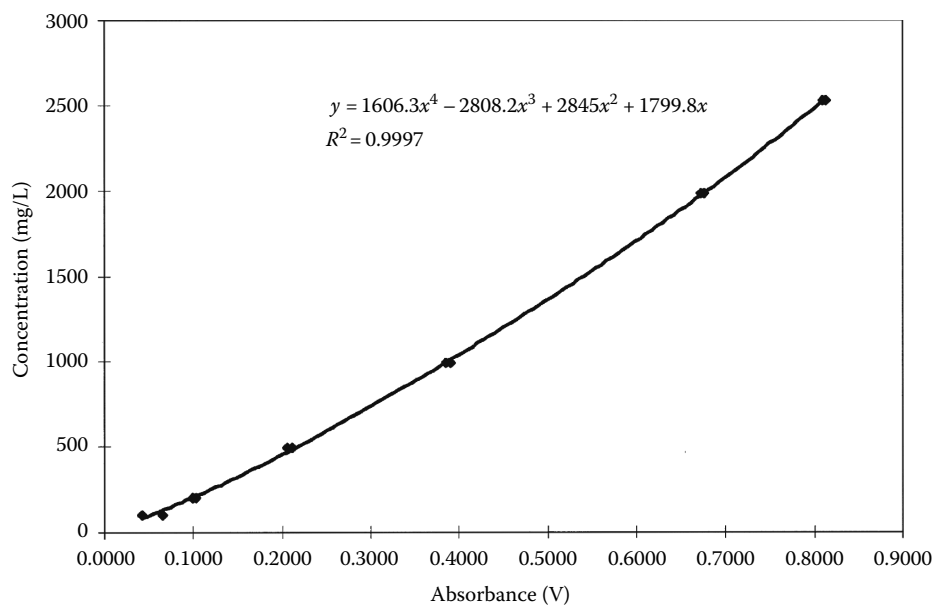


FIGURE 6.29 Example calibration graph for IR spectrophotometer. The IR spectrophotometer is calibrated by injecting known volumes of test article into a "closed-loop system" of known volume (5.64 L).

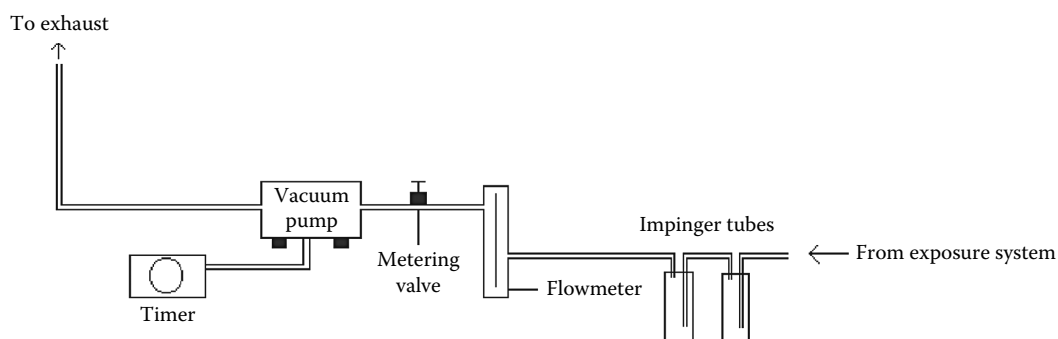


FIGURE 6.30 System for collection of impinger samples of test atmospheres for analytical evaluation. A vacuum pump is used to draw the air sample through tandem impingers (containing an appropriate solvent, e.g., water) from the exposure system.

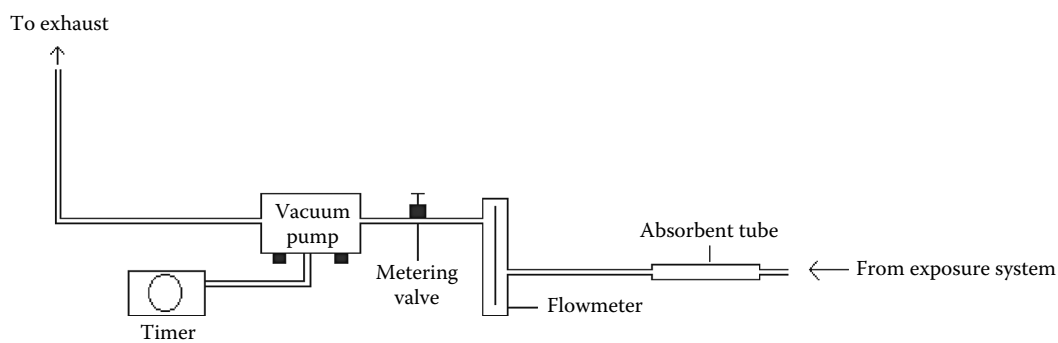


FIGURE 6.31 System for collection of absorbent tube samples of test atmospheres for analytical evaluation. A vacuum pump is used to draw the air sample through an absorbent tube (containing an appropriate medium, e.g., charcoal) from the exposure system.

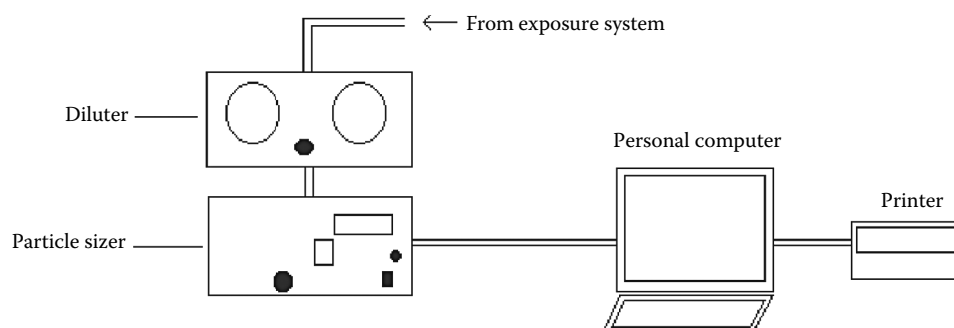


FIGURE 6.32 System for collection of aerosol samples of test atmospheres for particle size evaluation with the TSI aerodynamic particle sizer. An internal vacuum pump is used to draw the air sample through a diluter (100:1) and the particle sizer from the exposure system. The particle sizer uses a time-of-flight calibration based on the acceleration of particles through an orifice past a pair of laser beams.

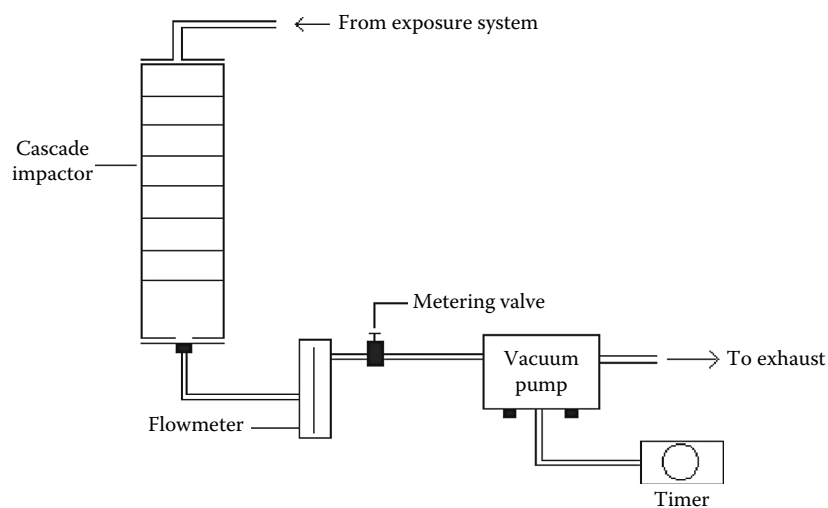


FIGURE 6.33 System for collection of aerosol samples of test atmospheres for particle size evaluation with a cascade impactor. A vacuum pump is used to draw the air sample through the multistage cascade impactor from the exposure system. The stages are individually assayed by gravimetric or analytical methodology.

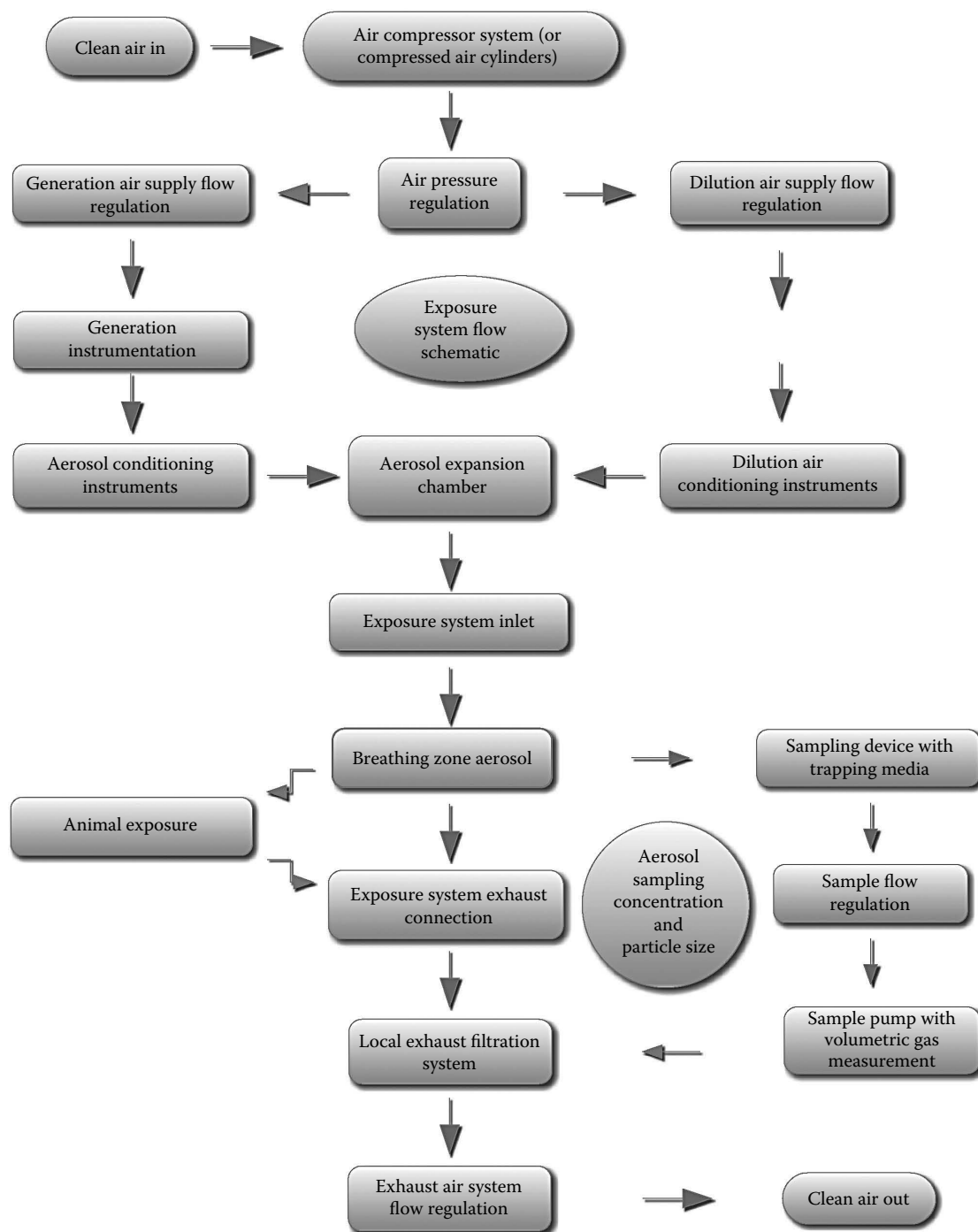


FIGURE 6.34 General flowchart for an exposure and sampling system for inhalation toxicology dosing.

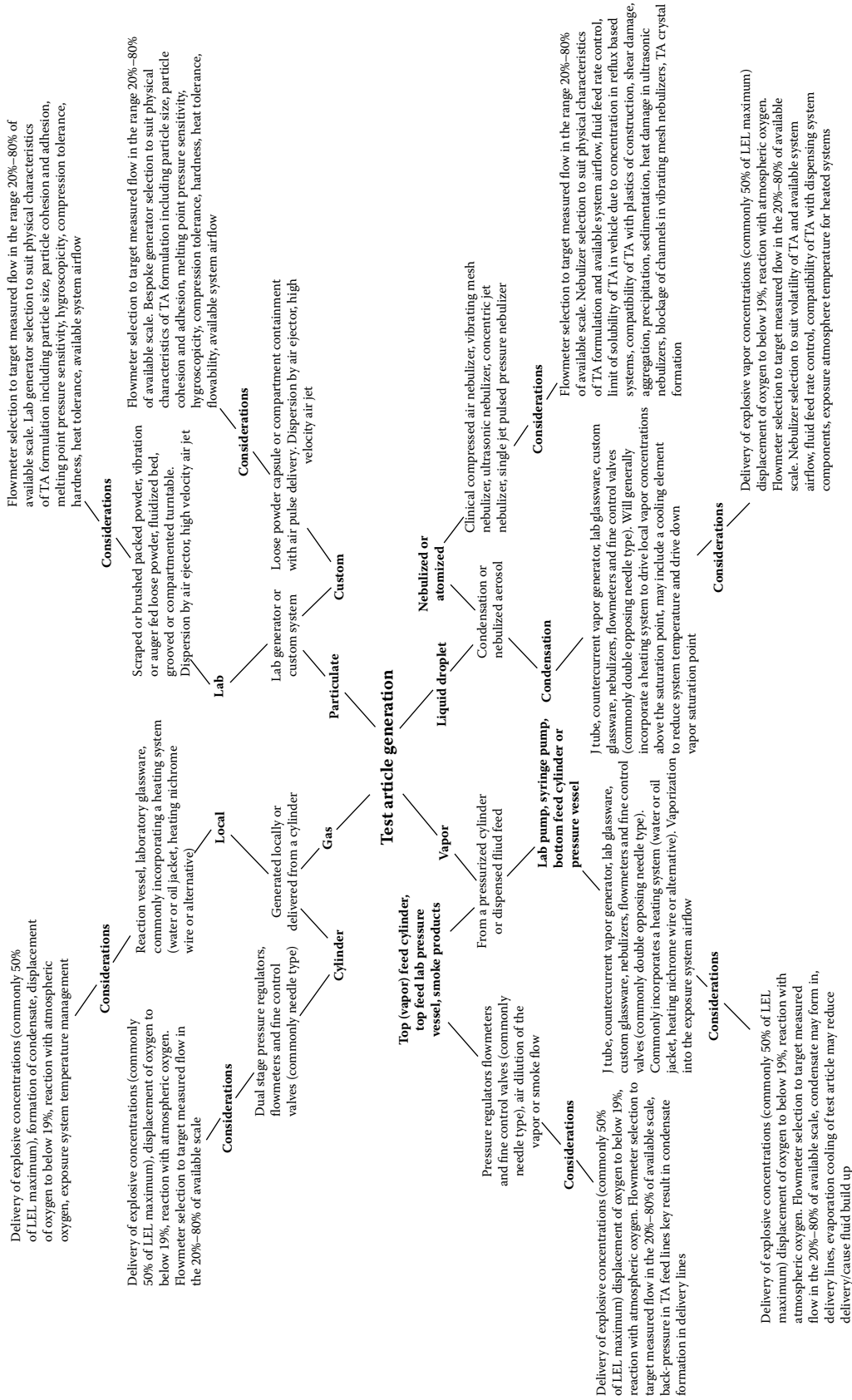


FIGURE 6.35 Test article generation selection considerations.

NANOPARTICLES

Although not commented upon in detail within this chapter, the interest and use of nanomaterials both from commercial and safety aspects are increasing. This is driving the development of alternative aerosol generation and characterization technologies for these materials.

Based on the recent guidance from the European Chemicals Agency (ECHA) and the RIP-oN3 (REACH Implementation Projects on Nanomaterials) project, it is proposed that particulate mass, number, and surface area are key metrics to be calculated. The main instruments available for measuring particulate mass that have not been mentioned within this chapter are the use of a tapered element oscillating microbalance (TEOM®), scanning mobility particle sizer (SMPS), or an electrical low pressure impactor (ELPI). For measuring particulate number, the main instruments are condensation particle counter (CPC), SMPS, ELPI, electron microscopy, or an optical particle counter (OPC). For particulate surface area, the main instruments are SMPS, ELPI, diffusion charger, or electron microscopy.

Considerations should be given based on the projected dimensions of the nanomaterials, particularly for HARN (high aspect ratio nanomaterials) test articles like multi-walled carbon nanotubes (MWCNT) or fibers.

Based on various sources within the literature, the use of standard methodology previously described for both generation and aerosol sampling appears to provide acceptable results.

EQUATIONS RELATED TO INHALATION TOXICOLOGY TESTING

1. The theoretical time that it takes a chamber to have an air change to a set of conditions is calculated as follows:

$$\text{Air change (min)} = \frac{V}{F},$$

where

V is the volume of the chamber (L)

F is the flow rate through the chamber (L/min)

2. The theoretical time that it takes a chamber to equilibrate to a set of conditions is known as T_x and is calculated as follows:

$$T_x(\text{min}) = K \times \frac{V}{F},$$

where

V is the volume of the chamber (L)

F is the flow rate through the chamber (L/min)

K is the exponential constant = 4.606 (99% equilibration) = 2.303 (90% equilibration)

3. The minimum flow rate for a nose-only exposure chamber is known as Q and is calculated as follows:

$$Q(\text{L/min}) = \text{animal number} \times \text{minute volume}$$

4. The volume-to-volume concentration of gas or vapor in air is calculated as follows:

$$\text{Concentration (ppm)} = \frac{\text{volume of vapor or gas } (\mu\text{L})}{\text{volume of air (L)}}$$

5. The conversion of concentration in ppm to weight-to-volume is calculated as follows:

$$\text{Concentration (mg/m}^3\text{)} = \text{Concentration (ppm)} \times \frac{MW}{24.5},$$

where

MW is the molecular weight (g/mol)

24.5 is the gas constant ($\mu\text{L}/\mu\text{mol}$) at 25°C and 760 mmHg

6. The conversion of concentration in weight-to-volume to ppm is calculated as follows:

$$\text{Concentration (ppm)} = \text{Concentration (mg/m}^3\text{)} \times \frac{MW}{24.5},$$

where

MW is the molecular weight (g/mol)

24.5 is the gas constant ($\mu\text{L}/\mu\text{mol}$) at 25°C and 760 mmHg

7. The concentration of a pure gas metered into an exposure chamber is calculated as follows:

$$\begin{aligned} \text{Concentration (ppm)} \\ = \frac{\text{Flow rate of gas (L/min)}}{\text{Flow rate of chamber (L/min)}} \times 10^6 \mu\text{L/L} \end{aligned}$$

8. The maximum attainable concentration in air for a volatile liquid is calculated as follows:

$$\begin{aligned} \text{Concentration (ppm)} \\ = \frac{\text{Vapor pressure (mmHg)}}{\text{Atmospheric pressure (mmHg)}} \times 10^6 \mu\text{L/L}, \end{aligned}$$

where atmospheric pressure = 760 mmHg at sea level

9. Haber's rule for concentration and response relationship is calculated as follows:

$$\text{Response} = C \times T,$$

where

C is the exposure concentration

T is the time of exposure

10. The nominal concentration for an exposure is calculated as follows:

$$\text{Nominal concentration (mg/m}^3\text{)} = \frac{W}{V} \times 1000 \text{ L/m}^3,$$

where

W is the quantity of test article consumed during the exposure (mg)

V is the volume of air through the chamber during the exposure (L)

Nominal concentration (ppm)

$$= \text{Concentration (mg/m}^3\text{)} \times \frac{24.5}{MW},$$

where

MW is the molecular weight (g/mol)

24.5 = gas constant ($\mu\text{L}/\mu\text{mol}$) at 25°C and 760 mmHg

11. The theoretical ventilation per minute of a resting mammal is known as V_m and may be calculated based on the Association of Inhalation Toxicologists (AIT) Working Party Recommendation for Standard Delivered Dose Calculation and Expression in

Non-Clinical Aerosol Inhalation Toxicology Studies with Pharmaceuticals⁴³:

$$RMV(\text{L/min}) = 0.608 \times BW(\text{kg})^{0.852},$$

where BW is the body weight of the animal (kg)

12. The theoretical dose level resulting from an inhalation exposure is calculated as follows:

$$\text{Dose (mg/kg)} = \frac{C \times RMV \times D \times DF}{BW}$$

where

C is the concentration (mg/L) in air inhaled

RMV is the respiratory minute volume (L/min)

D is the duration of exposure in minutes

DF is the deposition Fraction (generally assumed by regulatory agencies to be 100% for total respiratory tract deposition but 10% for small animals and 25% for large animals for pulmonary deposition)

BW is the body weight (kg)

GAS AND VAPOR CONVERSIONS (TABLE 6.72)

TABLE 6.72

Conversion Table for Gases and Vapors (Milligrams per Liter to Parts per Million, and Vice Versa; 25°C and 760 mmHg)^a

Molecular Weight	1 mg/L ppm	1 ppm mg/L	Molecular Weight	1 mg/L ppm	1 ppm mg/L	Molecular Weight	1 mg/L ppm	1 ppm mg/L
1	24,450	0.0000409	25	987	0.001022	49	499	0.002004
2	12,230	0.0000818	26	940	0.001063	50	489	0.002045
3	8,150	0.0001227	27	906	0.001104	51	479	0.002086
4	6,113	0.0001636	28	873	0.001145	52	470	0.002127
5	4,890	0.0002045	29	843	0.001186	53	461	0.002168
6	4,075	0.0002454	30	815	0.001227	54	453	0.002209
7	3,493	0.0002863	31	789	0.001268	55	445	0.002250
8	3,056	0.000327	32	764	0.001309	56	437	0.002290
9	2,717	0.000368	33	741	0.001350	57	429	0.002331
10	2,445	0.000409	34	719	0.001391	58	422	0.002372
11	2,223	0.000450	35	699	0.001432	59	414	0.002413
12	2,038	0.000491	36	679	0.001472	60	408	0.002554
13	1,881	0.000532	37	661	0.001513	61	401	0.002495
14	1,746	0.000573	38	643	0.001554	62	394	0.00254
15	1,630	0.000614	39	627	0.001595	63	388	0.00258
16	1,528	0.000654	40	611	0.001636	64	382	0.00262
17	1,438	0.000695	41	596	0.001677	65	376	0.00266
18	1,358	0.000736	42	582	0.001718	66	370	0.00270
19	1,287	0.000777	43	569	0.001759	67	365	0.00274
20	1,223	0.000818	44	556	0.001800	68	360	0.00278
21	1,164	0.000859	45	543	0.001840	69	354	0.00282
22	1,111	0.000900	46	532	0.001881	70	349	0.00286
23	1,063	0.000941	47	520	0.001922	71	344	0.00290
24	1,019	0.000982	48	509	0.001963	72	340	0.00294

TABLE 6.72 (continued)

Conversion Table for Gases and Vapors (Milligrams per Liter to Parts per Million, and Vice Versa; 25°C and 760 mmHg)^a

Molecular Weight	1 mg/L ppm	1 ppm mg/L	Molecular Weight	1 mg/L ppm	1 ppm mg/L	Molecular Weight	1 mg/L ppm	1 ppm mg/L
73	335	0.00299	124	197.2	0.00507	175	139.7	0.00716
74	330	0.00303	125	195.6	0.00511	176	138.9	0.00720
75	326	0.00307	126	194.0	0.00515	177	138.1	0.00724
76	322	0.00311	127	192.5	0.00519	178	137.4	0.00728
77	318	0.00315	128	191.0	0.00524	179	136.6	0.00732
78	313	0.00319	129	189.5	0.00528	180	135.8	0.00736
79	309	0.00323	130	188.1	0.00532	181	135.1	0.00740
80	306	0.00327	131	186.6	0.00536	182	134.3	0.00744
81	302	0.00331	132	185.2	0.00540	183	133.6	0.00748
82	298	0.00335	133	183.8	0.00544	184	132.9	0.00753
83	295	0.00339	134	182.5	0.00548	185	132.2	0.00757
84	291	0.00344	135	181.1	0.00552	186	131.5	0.00761
85	288	0.00348	136	179.8	0.00556	187	130.7	0.00765
86	284	0.00352	137	178.5	0.00560	188	130.1	0.00769
87	281	0.00356	138	177.2	0.00564	189	129.4	0.00773
88	278	0.00360	139	175.9	0.00569	190	128.7	0.00777
89	275	0.00364	140	174.6	0.00573	191	128.0	0.00781
90	272	0.00368	141	173.4	0.00577	192	127.3	0.00785
91	269	0.00372	142	172.2	0.00581	193	126.7	0.00789
92	266	0.00376	143	171.0	0.00585	194	126.0	0.00793
93	263	0.00380	144	169.8	0.00589	195	125.4	0.00798
94	260	0.00384	145	168.6	0.00593	196	124.7	0.00802
95	257	0.00389	146	167.5	0.00597	197	124.1	0.00806
96	255	0.00393	147	166.3	0.00601	198	123.5	0.00810
97	252	0.00397	148	165.2	0.00605	199	122.9	0.00814
98	249.5	0.00401	149	164.1	0.00609	200	122.3	0.00818
99	247.0	0.00405	150	163.0	0.00613	201	121.6	0.00822
100	244.5	0.00409	151	161.9	0.00618	202	121.0	0.00826
101	242.1	0.00413	152	160.9	0.00622	203	120.4	0.00830
102	239.7	0.00417	153	159.8	0.00626	204	119.9	0.00834
103	237.4	0.00421	154	158.8	0.00630	205	119.3	0.00838
104	235.1	0.00425	155	157.7	0.00634	206	118.7	0.00843
105	232.9	0.00429	156	156.7	0.00638	207	118.1	0.00847
106	230.7	0.00434	157	153.7	0.00642	208	117.5	0.00851
107	228.5	0.00438	158	154.7	0.00646	209	117.0	0.00855
108	226.4	0.00442	159	153.7	0.00650	210	116.4	0.00859
109	224.3	0.00446	160	152.8	0.00654	211	115.9	0.00863
110	222.3	0.00450	161	151.9	0.00658	212	115.3	0.00867
111	220.3	0.00454	162	150.9	0.00663	213	114.8	0.00871
112	218.3	0.00458	163	150.0	0.00667	214	114.3	0.00875
113	216.4	0.00462	164	149.1	0.00671	215	113.7	0.00879
114	214.5	0.00466	165	148.2	0.00675	216	113.2	0.00883
115	212.6	0.00470	166	147.3	0.00679	217	112.7	0.00888
116	210.8	0.00474	167	146.4	0.00683	218	112.2	0.00892
117	209.0	0.00479	168	145.5	0.00687	219	111.6	0.00896
118	207.2	0.00483	169	144.7	0.00691	220	111.1	0.00900
119	205.5	0.00487	170	143.8	0.00695	221	110.6	0.00904
120	203.8	0.00491	171	143.0	0.00699	222	110.1	0.00908
121	202.1	0.00495	172	142.2	0.00703	223	109.6	0.00912
122	200.4	0.00499	173	141.3	0.00708	224	109.2	0.00916
123	198.8	0.00503	174	140.5	0.00712	225	108.7	0.00920

(continued)

TABLE 6.72 (continued)

Conversion Table for Gases and Vapors (Milligrams per Liter to Parts per Million, and Vice Versa;
25°C and 760 mmHg)^a

Molecular Weight	1 mg/L ppm	1 ppm mg/L	Molecular Weight	1 mg/L ppm	1 ppm mg/L	Molecular Weight	1 mg/L ppm	1 ppm mg/L
226	108.2	0.00924	251	97.4	0.01027	276	88.6	0.01129
227	107.7	0.00928	252	97.0	0.01031	277	88.3	0.01133
228	107.2	0.00933	253	96.6	0.01035	278	87.9	0.01137
229	106.8	0.00937	254	96.3	0.01039	279	87.6	0.01141
230	106.3	0.00941	255	95.9	0.01043	280	87.3	0.01145
231	105.8	0.00945	256	95.5	0.01047	281	87.0	0.01149
232	105.4	0.00949	257	95.1	0.01051	282	86.7	0.01153
233	104.9	0.00953	258	94.8	0.01055	283	86.4	0.01157
234	104.5	0.00957	259	94.4	0.01059	284	86.1	0.01162
235	104.0	0.00961	260	94.0	0.01063	285	85.8	0.01166
236	103.6	0.00965	261	93.7	0.01067	286	85.5	0.01170
237	103.2	0.00969	262	93.3	0.01072	287	85.2	0.01174
238	102.7	0.00973	263	93.0	0.01076	288	84.9	0.01178
239	102.3	0.00978	264	92.6	0.01080	289	84.6	0.01182
240	101.9	0.00982	265	92.3	0.01084	290	84.3	0.01186
241	101.5	0.00986	266	91.9	0.01088	291	84.0	0.01190
242	101.0	0.00990	267	91.6	0.01092	292	83.7	0.01194
243	100.6	0.00994	268	91.2	0.01096	293	83.4	0.01198
244	100.2	0.00998	269	90.9	0.01100	294	83.2	0.01202
245	99.8	0.01002	270	90.6	0.01104	295	82.9	0.01207
246	99.4	0.01006	271	90.2	0.01108	296	82.6	0.01211
247	99.0	0.01010	272	89.9	0.01112	297	82.3	0.01215
248	98.6	0.01014	273	89.6	0.01117	298	82.0	0.01219
249	98.2	0.01018	274	89.2	0.01121	299	81.8	0.01223
250	97.8	0.01022	275	88.9	0.01125	300	81.5	0.01227

Sources: Clayton, G.D. and Clayton, F.E., Eds., *Patty's Industrial Hygiene and Toxicology*, 4th edn., John Wiley & Sons, New York, 1991. With permission; Fieldner, A.C. et al., Gas masks for gasses met in fighting fires, U.S. Bureau of Mines, Technical Paper No. 248, 1921.

^a For example - for a gas of molecular weight of 100, 1 mg/L = 244.5 ppm and 1 ppm = 0.00409 mg/L.

REFERENCES

1. Tyler, W.S. and Julian, M.D., Gross, and subgross anatomy of lungs, pleura, connective tissue septa, distal airways and structural units, in *Treatise On Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 4.
2. Schreider, J.P., Nasal airway anatomy and inhalation deposition in experimental animals and people, in *Nasal Tumors in Animals and Man, Vol. III, Experimental Nasal Carcinogenesis*, Reznik, G. and Stinson, Eds., CRC Press, Boca Raton, FL, 1983, pp. 1-26.
3. Pinkerton, K.E., Gehr, P., and Crapo, J.D., Architecture and cellular composition of the airblood barrier, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 11.
4. Tenney, S.M. and Bartlett, D., Comparative quantitative morphology of the mammalian lung: Trachea, *Respir. Physiol.*, 3, 130, 1967.
5. Gehr, P., Mwangi, D.K., Ammann, A., Maloiy, G.M.O., Taylor, C.R., and Weibel, E.R., Design of the mammalian respiratory system, V, scaling, morphometric pulmonary diffusing capacity to body mass: Wild and domestic mammals, *J. Respir. Physiol.*, 44, 61, 1981.
6. Jones, J.H. and Longworth, K.E., Gas exchange at rest and during exercise in mammals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 19.
7. Li-Fock, S.J. and Kaplowitz, M.R., Pleural liquid thickness *in situ* by light microscopy in five mammalian species, *J. Appl. Physiol.*, 59, 603, 1985.
8. Pinkerton, K.E., Mercer, R.R., Plopper, C.G., and Crapo, J.D., Distribution of injury and microdosimetry of ozone in the ventilatory unit of the rat, *J. Appl. Physiol.*, 73, 817, 1992.
9. Miller, F.J., Overton, J.H., Kimbell, J.S., and Russell, M.L., Regional respiratory tract absorption of inhaled reactive gasses, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993, Chapter 18.

10. Crapo, J.D., Young, S.L., Fram, E.K., Pinkerton, K.E., Barry, B.E., and Crapo, R.O., Morphometric characteristics of cells in the alveolar region of mammalian lungs, *Am. Rev. Respir. Dis.*, 128, 542, 1983.
11. Phalen, R.E., *Inhalation Studies: Foundations and Techniques*, CRC Press, Boca Raton, FL, 1984.
12. Schreider, J.P., Comparative anatomy and function of the nasal passages, in *Toxicology of the Nasal Passages*, Barrow, C.S., Ed., Hemisphere Publishing, New York, 1986, Chapter 1.
13. Plopper, C.G., Mariassy, A.T., Wilson, D.W., Alley, J.L., Nishio, S.J., and Nettesheim, P., Comparison of nonciliated tracheal epithelial cells in six mammalian species: Ultrastructure and population densities, *Exp. Lung. Res.*, 5, 281, 1983.
14. St. George, J.A., Harkema, J.R., Hyde, D.M., and Plopper, D.G., Cell populations and structural function relationships of cells in the airways, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993, Chapter 4.
15. Mariassy, A.T., Epithelial cells of the trachea and bronchi, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 6.
16. Plopper, C.G. and Hyde, D.M., Epithelial cells of bronchioles, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 8.
17. Stone, K.C., Mercer, R.R., Gehr, P., Stockstill, B., and Crapo, J.D., Allometric relationships of cell numbers and size in the mammalian lung, *Am. J. Respir. Cell. Mol. Biol.*, 6, 235, 1992.
18. Snipes, M.B., Long term retention and clearance of particles inhaled by mammalian species, *Crit. Rev. Toxicol.*, 20, 174, 1989.
19. Boggs, D.F., Comparative control of respiration, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 20.
20. Mauderly, J.L., The effect of age on respiratory function of Fischer-344 rats, *Exp. Aging Res.*, 8, 31, 1982.
21. Sahebajani, H., Aging of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 21.
22. Mauderly, J.L., Ventilation, lung volumes and lung mechanics of young adult and old Syrian hamsters, *Exp. Aging Res.*, 5, 497, 1979.
23. Lai, Y-L., Comparative ventilation of the normal lung, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 17.
24. Johanson, W.G., Jr. and Pierce, A.K., Lung structure and function with age in normal rats and rats with papain emphysema, *J. Clin. Invest.*, 52, 2921, 1973.
25. Henderson, R.F., Bronchoalveolar lavage: A tool for assessing the health status of the lung, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, 1989, Chapter 15.
26. McDermott, M.R., Befus, A.D., and Bienenstock, J., The structural basis for immunity in the respiratory tract, *Int. Rev. Exp. Pathol.*, 23, 47, 1982.
27. Murray, M.J. and Driscoll, K.E., Immunology of the respiratory system, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 37.
28. Schlesinger, R.B., Deposition and clearance of inhaled particles, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, 1989, Chapter 6.
29. Valberg, P.A. and Blanchard, J.D., Pulmonary macrophage physiology: Origin, motility, endocytosis, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 36.
30. Stöber, W., McClellan, R.O., and Morrow, P.E., Approaches to modeling disposition of inhaled particles and fibers in the lung, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993, Chapter 19.
31. Wolff, R.K., Mucociliary function, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 35.
32. Klimisch, H.-J., Bretz, R., Doe, J.E., and Purser, D.A., Classification of dangerous substances and pesticides in the European economic community directives: a proposed revision of criteria for inhalation toxicity, *Reg. Toxicol. Pharmacol.*, 7, 21, 1987.
33. Kane, L.E., Barrow, C.S., and Alaire, Y., A short-term test to predict acceptable levels of exposure to airborne sensory irritants, *Am. Ind. Hyg. Assoc. J.*, 40, 207, 1979.
34. Boggs, D.F. and Tenney, S.M., Scaling respiratory pattern and respiratory drive, *Respir. Physiol.*, 58, 245, 1984.
35. Schlesinger, R.B., Ben-Jebria, A., Dahl, A.R., Snipes, M.B., and Ultman, J., Disposition of inhaled toxicants, in *Handbook of Human Toxicology*, Massaro, E.J., Ed., CRC Press, Boca Raton, FL, 1997, Chapter 12, pp. 493–550.
36. Costa, D.L., Tepper, J.S., and Raub, J.A., Interpretations and limitations of pulmonary function testing in small laboratory animals, in *Treatise on Pulmonary Toxicology, Vol. 1, Comparative Biology of the Normal Lung*, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, Chapter 22.
37. Hahn, F.F., Chronic inhalation bioassays for respiratory tract carcinogenesis, in *Toxicology of the Lung*, 2nd edn., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Raven Press, New York, 1993, Chapter 16.
38. Schlessinger, R.B., The interaction of inhaled toxicants with respiratory tract clearance mechanisms, *Crit. Rev. Toxicol.*, 20, 297, 1990.
39. Cheng, Y.-S. and Moss, O.R., Inhalation exposure systems, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, 1989, Chapter 1.
40. Fieldner, A.C., Katz, S.H., and Kinney, S.P., Gas masks for gasses met in fighting fires, U.S. Bureau of Mines, Technical Paper No. 248, 1921.
41. Clayton, G.D. and Clayton, F.E., Eds., *Patty's Industrial Hygiene and Toxicology*, 4th edn., John Wiley & Sons, New York, 1991.
42. Moss, O.R. and Cheng, Y.-S., Generation and characterization of test atmospheres: particles, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing, New York, 1989, Chapter 3.
43. Alexander, D.J. et al., Association of Inhalation Toxicologists (AIT) working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. *Inhal. Tox.*, 20, 1179–1189, 2008.

7 Neurotoxicology

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INTRODUCTION

Neurotoxicity is defined as an adverse change in the structure or function of the nervous system following exposure to a chemical, biological, or physical agent. An important concept in assessing neurotoxicity is that of the selective vulnerability of the nervous system to injuries, which is based on the structural complexity and cellular and molecular heterogeneity of the nervous system. Neurotoxicants can preferentially affect specific neuroanatomical regions and, within those regions, the target can be a specific type of neuronal or glial cell. Based on the selective vulnerability of the nervous system, neurotoxic injuries have been classified based on the neuronal structure or function affected. Examples of neurotoxicants responsible for each type of injury are presented in Tables 7.1 through 7.4.^{1-7,9-24}

1. *Neuronopathy*: The neurotoxic agent directly affects the cell body of the neuron (perikaryon). This often leads to the death of the neuron, including its cytoplasmic extensions (axons and dendrites) as well as the protective myelin sheath, and is irreversible.
2. *Axonopathy*: In this type of injury, the primary site of toxicity is the axon and it produces an effect that is the chemical equivalent of an axonal transection. This generally leads to the degeneration of the axon distal to the transection point, followed by the secondary degeneration of the myelin sheath. The cell body itself may be spared. Axonopathies have

been further classified as “central–peripheral distal axonopathy” to indicate those injuries primarily involving the distal ends of long axons; “dying back” neuropathy that indicates that the distal axon progressively degenerates from the end of the axon back to the cell body; and “central–peripheral proximal axonopathy” in which the primary degeneration of the axon takes place proximal to the spinal cord. Since peripheral axons can regenerate, partial or complete recovery can occur after they are injured. This is generally not true for injuries to axons in the central nervous system (CNS).

3. *Myelinopathy*: Myelin is formed by oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS) and provides the electrical insulation for axonal neurotransmission. Injuries to the myelin sheath lead to slow or aberrant transmissions along the affected axon. Neurotoxicants may produce myelinopathy by separating the myelin lamellae, resulting in intramyelinic edema, or by causing the loss of myelin (demyelination).
4. *Neurotransmitter effects*: Some substances may produce functional neurological changes by impairing the process of neurotransmission without producing structural changes in the neuron. These neurotoxicants may interrupt transmission of impulses, block or accentuate synaptic transmissions, or interfere with the second-messenger system.

TABLE 7.1
Examples of Chemicals Producing Neuronopathy

Neurotoxicant	Functional Effects	Morphological Effects	References
Aluminum	Learning and memory deficits, tremor, incoordination, weakness, ataxia, seizures	Neurofibrillary aggregates, degeneration of cortical cells	[1,2]
Domoic acid	Rigidity, stereotypy, loss of balance, memory loss, confusion, seizures	Neuronal loss, hippocampus, and amygdala; layers 5 and 6 of neocortex	[3–6]
Doxorubicin	Ataxia	Degeneration of dorsal root ganglion cells, degeneration of peripheral nerves	[7]
Manganese	Parkinson-like disorder (loss of facial expression, tremors, rigidity, gait disturbances, ataxia)	Degeneration of basal ganglia, especially globus pallidus and striatum	[8,9]
Methylmercury	Constriction of visual fields, cerebellar ataxia, paresthesias	Neuronal degeneration of the visual cortex, cerebellum, and dorsal root ganglia	[10]
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	Parkinsonism (loss of facial expression, tremors, rigidity, gait disturbances, ataxia)	Neuronal degeneration in the substantia nigra	[11,12]
Trimethyltin	Aggression, hyperirritability, hyperreactivity, muscle weakness, tremors, convulsions	Necrosis of hippocampal neurons; also brain stem and spinal cord (mice) and areas of the limbic system (rats)	[9,13]

TABLE 7.2
Examples of Chemicals Producing Axonopathy

Neurotoxicant	Functional Effects	Morphological Effects	References
Acrylamide	Ataxia, tremor, possible paralysis, weakness, lethargy, paresthesia, decreased pin-prick sensation	Central–peripheral distal axonopathy (“dying-back” type)	[14,15]
Carbon disulfide	Sensory deficits and motor weakness	Neurofilament-filled axonal swellings in the distal axon, leading to axonal degeneration	[16]
Hexane	Sensory deficits and motor weakness	Neurofilament-filled axonal swellings in the distal axon, leading to axonal degeneration	[16]
3,3'-Iminodipropionitrile (IPDN)	Sensory deficits and motor weakness	Neurofilament-filled axonal swellings in the proximal axon, atrophy of distal axon	[16,17]
Pyridinethione	Sensory deficits and motor weakness	Axonal swellings containing tubular and vesicular structures in the distal axon, leading to axonal degeneration	[17]
Tri-ortho-cresyl phosphate (TOCP)	Delayed peripheral neuropathy, spasticity	Central–peripheral distal axonopathy (peripheral nerves and spinal cord)	[17]

TABLE 7.3
Examples of Chemicals Producing Myelinopathy

Neurotoxicant	Functional Effects	Morphological Effects	References
Acetyl-ethyl-tetramethyl-tetralin (AETT)	Hyperexcitability, limb weakness, ataxia, learning impairment	Intramyelinic edema in CNS and PNS; neuronal accumulation of ceroid-like pigments	[18,19]
Cuprizone	Encephalopathy	Intramyelinic edema in CNS; degeneration of myelinated nerves in PNS	[20]
Hexachlorophene	Irritability, confusion, seizures, impaired vision, coma and death possible, especially in infants	Intramyelinic edema in CNS and PNS, vacuolation of white matter in CNS, degeneration of optic nerve	[21,22]
Tellurium	Hind limb weakness	Demyelination in PNS	[23]
Triethyltin	Headache, visual disturbances, vertigo, paralysis	Intramyelinic edema in CNS, brain swelling	[24]

Note: CNS, central nervous system; PNS, peripheral nervous system.

TABLE 7.4
Examples of Chemicals Producing Effects
on Neurotransmission

Neurotoxicant	Functional Effect	Neurotransmission Effect
Amphetamine	Hyperactivity, restlessness, dizziness, tremor, irritability, stereotyped behavior	Interacts with catecholamine neurons to increase levels of norepinephrine and dopamine
Benzodiazepines	Motor incoordination, ataxia, mental confusion, lethargy	Potential of inhibitory effects of γ -aminobutyric acid (GABA)
Physostigmine	Cholinergic effects, example, miosis, tremors, confusion, excessive salivation and lacrimation, lethargy	Increases acetylcholine levels at nerve terminals by inhibition of acetylcholinesterase

REGULATORY GUIDELINES

It is clear that neurotoxicants can elicit a broad spectrum of biochemical, structural, and functional abnormalities. This diversity must be taken into consideration in screening unknown chemicals for the potential to produce neurotoxicity. The approach taken by governmental regulatory agencies to address this diversity of effects combines neurobehavioral testing and neuropathology evaluations.

Behavior is the adaptive response of an organism to internal or external stimuli and, as such, represents the integrated end product of multiple neuronal subsystems. Thus, evaluation of behavior can serve as an indicator of the status of the functional components of the nervous system. In addition, since behavioral testing is noninvasive, it can provide a longitudinal assessment of the neurotoxic effects of an agent, including persistence, delayed onset, or recovery. Typically, a functional observational battery (FOB) is employed to assess a wide range of neurobiological functions, including sensory, motor, and autonomic components and the measurement of motor activity using an automated device.

Neuropathology evaluations should include tissues collected from all major regions of the nervous system. If neuropathological lesions are detected using standard stains, special stains may be used to characterize the abnormality further and determine the no-observable-effect level.

Tables 7.5 through 7.7 list neurotoxicity screening guidelines for the US Environmental Protection Agency (EPA), the Organization for Economic Cooperation and Development (OECD), and the US Food and Drug Administration (FDA) Redbook 2000, respectively. These represent tier-1 testing; additional morphological, chemical, or physiological evaluations may be necessary to provide a complete evaluation of neurotoxicity. Some of the guidelines apply to stand-alone neurotoxicity studies whose specific purpose is to evaluate neurotoxicity. These studies can be combined with any other toxicity study,

as long as the basic requirements of both types of studies are met. No specific protocol requirements have been issued for neurotoxicity testing of pharmaceuticals; testing requirements of drug candidates are generally determined on a case-by-case basis.

All of the guidelines generally include the following elements:

Animal species: The preferred species is the laboratory rat. Other species, such as the mouse or dog, may be used under some circumstances, but neurobehavioral tests have to be adapted to the species used.

Age: Young adult rats, approximately 6 weeks old, at the start of the study.

Number of animals: At least 20 rats (10 males and 10 females) per dose or control group are generally used for neurobehavioral testing. Of those 20 animals, 10 (5 males and 5 females) are used for terminal neuropathology, if required.

Treatment and control groups: At least three doses and a vehicle control are recommended.

Dose selection: The high dose should produce significant neurotoxic effects or other clearly toxic systemic effects. The high dose need not be >2 g/kg body weight for acute studies and 1 g/kg body weight for subchronic studies. The incidence of mortality in the high dose should not be such that it precludes a meaningful evaluation of the data. The mid and low doses should be fractions of the high dose to demonstrate any dose-related responses. There should be minimal or no effects at the low dose.

Route of exposure: Selection of the route of exposure may be based on several criteria, including most likely route of human exposure, bioavailability, the likelihood of observing effects, practical difficulties, and the likelihood of observing nonspecific effects.

Specialized neuropathology: See Section "Neuropathology."

TABLE 7.5
US EPA Regulatory Guidelines for Neurotoxicity Testing

Study Type	Guideline No.	Date	Neurobehavioral Assessment	Specialized Neuropathology
Acute neurotoxicity screen	OPPTS 870.6200	August 1998	Yes	Yes
Subchronic neurotoxicity screen	OPPTS 870.6200	August 1998	Yes	Yes
90-d oral toxicity in rodents	OPPTS 870.3100	August 1998	Yes	No
Chronic toxicity in rodents (12 mo)	OPPTS 870.4100	August 1998	Yes	No
21/28-d dermal toxicity in rodents	OPPTS 870.3200	August 1998	Yes	No
90-d dermal toxicity in rodents	OPPTS 870.3250	August 1998	Yes	No
90-d inhalation toxicity in rodents	OPPTS 870.3465	August 1998	Yes	No

TABLE 7.6
OECD Regulatory Guidelines for Neurotoxicity Testing

Study Type	Guideline No.	Date	Neurobehavioral Assessment	Specialized Neuropathology
Acute neurotoxicity study in rodents	424	July 1997	Yes	Yes
28-d neurotoxicity study in rodents	424	July 1997	Yes	Yes
90-d neurotoxicity study in rodents	424	July 1997	Yes	Yes
Chronic neurotoxicity study in rodents	424	July 1997	Yes	Yes
28-d oral toxicity study in rodents	407	July 1995	Yes	No
90-d oral toxicity study in rodents	408	September 1998	Yes	No

TABLE 7.7
US FDA Redbook 2000 Regulatory Guidelines for Neurotoxicity Testing

Study Type	Guideline No.	Date	Neurobehavioral Assessment	Specialized Neuropathology
Short-term toxicity studies with rodents	IV.C.3	November 2003	Yes	No
Subchronic toxicity studies with rodents	IV.C.4.a	November 2003	Yes	No
Neurotoxicity studies with rodents	IV.C.10	July 2000	Yes	Yes

FOB EVALUATIONS IN RATS

The FOB comprises a series of assessments designed to measure motor, sensory, and autonomic function, typically in the rat (Table 7.8). These assessments are conducted progressively at the following times: (1) when the rat is in its home cage; (2) while the rat is being handled and held during removal from the cage; (3) while the rat is moving freely in an open field; and (4) during manipulative tests. Since many of the assessments in the FOB are subjective, it is important to be aware of potential sources of bias. Factors to consider in controlling bias include the following:

1. Since many of the end points used in the FOB involve subjective ranking, it is necessary to have a scoring system with explicitly defined scales. An example of such a scoring system is presented

in Table 7.9; similar scoring systems have been published.^{25–28} In general, testing proceeds from evaluations that require no interaction with the subject (home cage observations) to those that require active manipulation of the subject (e.g., grip strength, landing foot splay).

2. Observers who conduct the FOB should be carefully trained to recognize different types of abnormal behavior in the rat as well as the importance of handling the animals in a gentle and consistent manner. The training is done most effectively using rats that have been treated with known neurotoxins to illustrate the differences between control and treated animals. Training can often be combined with the collection of positive control data, used to provide evidence that the observational methods can detect major neurotoxic end points.

Some of the chemicals used for this purpose are included in Table 7.10.²⁹ Chemicals that produce transient neurological effects are preferred for training and collection of positive control data, as compared with chemicals that produce irreversible structural damage, since animals can then be used more than once and the number of animals used in training can be reduced.

3. A training DVD and reference manual for conducting an FOB have been produced under the sponsorship of the US EPA and the American Industrial Health Council.*²⁹ These training materials are very useful in describing an array of abnormal behaviors and are an invaluable adjunct to in-life training.
4. The same observer should be used as much as possible to evaluate all the animals in a study. Since that is not always feasible, some demonstration of interobserver reliability is needed.
5. Observers who are conducting the FOB should be "blinded," that is, unaware of the animal's assignment to a particular treatment group. In addition, the order of testing the animals should be randomized so that there is no discernible pattern to the order in which treatment levels are tested.
6. Efforts should be made to ensure that variations in environmental test conditions are minimal and are not systematically related to treatment. These factors include lighting, temperature, humidity, noise level, odors, and environmental distractions. Others factors that should be controlled arise from the need to stagger dosing and evaluations over several days because of the length of time required to assess each animal in a study. In a typical study with four groups and 10 rats/sex/group, testing of the 80 rats occurs over a 4-day period such that 10 males and 10 females are tested each day. Time of day in which the FOB is conducted must therefore be controlled. Furthermore, the time of testing must be balanced against sex and treatment group. Thus, five animals from each dose group (two to three rats/sex/dose) must be tested in the same time frame on each day of testing.

Interpretation of the results of the FOB is complicated by the large amount of data that is generated and also by the different types of data that are collected. An examination of the scoring criteria shows that the data falls into the following types: continuous data (e.g., grip strength and landing foot splay), ranked or ordinal data (e.g., salivation, air-righting reflex), and descriptive or nominal data (e.g., home cage posture). One approach that has been presented to summarize FOB data and make it more accessible for

statistical analysis uses the concept of functional domains of neurobehavior.^{30,31} In this approach, certain measures of the FOB are grouped together under domains that represent broad neurobiological categories (Table 7.11).³⁰ These data can then provide profiles of effects for different chemicals. This approach has the advantage of not placing a disproportionate emphasis in interpreting the data on any one test measure. Examples of this type of analysis are presented in Table 7.12³¹ for acrylamide, dichlorodiphenyl trichloroethane (DDT), and parathion.

TABLE 7.8
Measures Included in the FOB

1. Assessment of the signs of autonomic function, including, but not limited to
 - a. Ranking of the degree of lacrimation and salivation
 - b. Presence or absence of piloerection and exophthalmus
 - c. Ranking or count of urination and defecation
 - d. Pupillary size, such as constriction of the pupil in response to light
 - e. Degree of palpebral closure
2. Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements, both in the home cage and the open field
3. Ranking of reactivity to general stimuli, such as removal from the cage or handling, with a range of severity scores from no reaction to hyperactivity
4. Ranking of the general level of activity during observation in the open field, with a range of severity scores from unresponsive to hyperactive
5. Description and incidence of posture and gait abnormalities observed in the home cage and open field
7. Ranking of any gait abnormalities, with a range of severity scores from none to severe
7. Forelimb and hind limb grip strength using an objective procedure
8. Quantitative measure of landing foot splay
9. Sensorimotor responses to stimuli of different modalities to detect gross sensory deficits; pain perception may be assessed by a ranking or measure to a tail pinch, tail flick, or hot plate; audition may be assessed by the response to a sudden sound, example, click or finger-snap
10. Body weight
11. Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypes), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data
12. Additional measures that may also be included are as follows:
 - a. Count of rearing activity on the open field
 - b. Ranking of righting ability
 - c. Body temperature
 - d. Excessive or spontaneous vocalization
 - e. Alterations in rate and ease of respiration, example, rales or dyspnea
 - f. Sensorimotor responses to visual or proprioceptive stimuli

* The training manual in Word format and the DVD are available upon request from Dr. Virginia Moser at USEPA, Neurotoxicology, HERL, MD-74B, Research Triangle Park, NC 27711, Fax (919) 541-5075; E-mail: moser.ginger@epamail.epa.gov.

Source: US Environmental Protection Agency, Health Effects Test Guidelines, OPPTS 870.6200, Neurotoxicity Screening Battery.

TABLE 7.9
Sample FOB Evaluation Scheme with Descriptions of Evaluations

- **Equipment needed**
 - White noise generator
 - Sound and light meters
 - Stopwatch
 - Laboratory cart
 - Absorbent paper
 - Penlight
 - Forceps
 - Pencil or other blunt object
 - Nalgene or other container, approximately 18 × 15 × 5 in., filled with clean bedding (for air righting)
 - Grip strength meters
 - A plastic container of slightly dampened sand, placed on a laboratory cart or other flat work surface (or ink pad or nontoxic paint and paintbrush) (for landing foot splay)
 - Ruler
 - Dog training “clicker” or similar
- **Room preparation**
 - Turn on the white noise generator, set at 55–65 dB
 - Monitor and record: room temperature, humidity, light intensity (lumens)
 - Fill Nalgene or other container with bedding; using a ruler, measure 12 in. above the top of the bedding and mark this spot on the wall
 - Measure 12 in. above the sand surface in the plastic container (or above flat surface covered with paper if using ink or paint)
 - Calibrate the grip strength meters
 - Randomize animals (performed by technician other than the one conducting the FOB)
- **Examination procedure**
 - Record the initiation time of the FOB for each animal
 - Record all of the observations in the appropriate columns of the FOB form
 - Explain any abnormal behavior in the comments section as needed
- **In-cage observations**
 - Posture*—Score as follows:
 - 1 = Sitting or standing normally
 - 2 = Rearing
 - 3 = Curled up
 - 4 = Hunched
 - 5 = Flattened
 - 6 = Lying on side
 - Abnormal motor movements*
 - 1 = No abnormal movements
 - 2 = Tremors
 - 3 = Fasciculation
 - 4 = Convulsions
 - 5 = Stereotypy. Description required (e.g., circling, sniffing, licking, grooming)
 - 6 = Other
 - Palpebral closure*—Score as follows:
 - 1 = Eyelids open
 - 2 = Eyelids slightly drooping
 - 3 = Eyelids half closed
 - 4 = Eyelids completely closed

TABLE 7.9 (continued)
Sample FOB Evaluation Scheme with Descriptions of Evaluations

- **Handling evaluations**—Remove the animal from its cage and evaluate for
 - Ease of removal*—Score as follows:
 - 1 = Atypically docile, with minimal awareness
 - 2 = Easy; shows awareness but no avoidance or resistance
 - 3 = Moderately difficult; some resistance or avoidance but shows no aggression
 - 4 = Difficult; considerable resistance or avoidance, minimal signs of fear or aggression
 - 5 = Very difficult; runs around cage, is hard to pick up, attempts to bite or attack
 - Reactivity to handling*—Score as follows:
 - 1 = Atypically docile, with minimal awareness
 - 2 = Easy; shows awareness, but little or no resistance
 - 3 = Slightly awkward, some struggling or squirming
 - 4 = Difficult, noticeable struggling making handling difficult
 - 5 = Very difficult; excessive struggling and aggression
 - Lacrimation*—Score as follows:
 - 1 = No lacrimation
 - 2 = Slight discharge (fluid on fur at the corner of the eye)
 - 3 = Moderate discharge (fluid on fur just under eye)
 - 4 = Marked discharge (fluid on facial fur)
 - Salivation*
 - 1 = No salivation
 - 2 = Slight salivation (damp around mouth)
 - 3 = Moderate salivation (noticeably wet around mouth)
 - 4 = Marked salivation (wet on the chin and extending to the throat)
 - Exophthalmos*
 - 1 = Absent (normal)
 - 2 = Present; eyes protrude abnormally
 - Vocalization*
 - 1 = None
 - 2 = Vocalizing softly
 - 3 = Vocalizing moderately
 - 4 = Vocalizing loudly/aggressively
 - Piloerection*
 - 1 = Absent (normal)
 - 2 = Present (coat erect on dorsal surface)
 - Coat*
 - 1 = Normal, well groomed
 - 2 = Slightly matted, unkempt or stained
 - 3 = Moderately matted, unkempt or stained
 - 4 = Markedly matted, unkempt or stained
- **Weigh the animal and record the body weight**
- **Open field observations**
 - Place the animal on a clean laboratory cart for at least 2 min
 - Replace absorbent paper on the cart between each animal
 - Gait* (abnormalities in gait are graded and described)
 - U = Unable to assess (insufficient locomotion)
 - 1 = Normal
 - 2 = Slightly abnormal
 - 3 = Moderately abnormal
 - 4 = Markedly abnormal

TABLE 7.9 (continued)
Sample FOB Evaluation Scheme with Descriptions of Evaluations

Common gait abnormalities

- A = Ataxia, uncoordinated movement, excessive sway, rock, or lurch
- F = Body drags or is flattened (animal's ventral surface makes contact with the cart surface)
- E = Elevated (body held high off the cart surface; walking on tiptoes)
- S = Limbs splayed or dragging; unable to support weight (specify hind limbs or forelimbs)

Locomotion (ability to move from place to place)

- 1 = Normal (animal moves easily around open field)
- 2 = Increased movement (animal moves mostly continuously, rarely stopping to sniff or groom)
- 3 = Decreased movement (reduced movement around the field; movements may be sluggish)
- 4 = None (animal does not move around the field)

Arousal (evaluated after observing indicators of exploration, including locomotion, rearing, grooming, whisking, and sniffing)

- 1 = Normal; alert with sniffing and exploratory movements
- 2 = Low; slight stupor, some head or body movements
- 3 = Very low; stupor, little or no responsiveness to the environment
- 4 = High; slight excitement, tense, sudden darting, or freezing
- 5 = Very high; hyperalert, sudden boost of running or movement

Abnormal motor movements or bizarre behavior (e.g., twitches, tremors, convulsions, fasciculation, stereotypy)

- 1 = None
- 2 = Present

Relevant information (e.g., limbs affected) will be recorded.

Convulsions will be classified as tonic or clonic in the comment section. Any other unusual or abnormal movement or behavior will be described. This includes, but is not limited to, head weaving (movement from side to side), head bobbing (up and down movement), head tilt, backward movement, excessive or repetitive actions (stereotyped, e.g., sniffing, grooming, licking, circling), straub tail (tail is stiff and held in vertical position for extended periods).

Record the number of pools of urine (if polyuria [apparently uncontrolled urination] is noted, record an "X")

• **Reflex assessments**

Response to visual stimulus (evaluate by approaching the animal's head with a blunt object and holding the object 1–2 in. from the animal's head for a few seconds)—Score as follows:

- 1 = Normal, slowly approaches, sniffs, and/or turns away
- 2 = No reaction
- 3 = Abnormally fearful or aggressive reaction

Response to auditory stimulus (e.g., a click or finger snap above and behind the animal's head will be used to assess audition)

- 1 = Normal; flinches and/or flicks ears
- 2 = No reaction
- 3 = Exaggerated; jumps, bites

Pupil response to light (beam of a penlight is brought in from the side of the head, and the pupil is observed; lights are turned off as necessary).

Each eye is tested separately

- 1 = Pupil constricts normally
- 2 = Pupil size does not change
- 3 = Miosis
- 4 = Mydriasis

TABLE 7.9 (continued)
Sample FOB Evaluation Scheme with Descriptions of Evaluations

Pinna reflex (to assess the sensitivity to a light touch, inside the ear, with a fine object)

- 1 = Ear flattens against head
- 2 = Animal shakes head
- 3 = No response

Proprioception (gently restrain the animal on a horizontal surface by grasping the thorax; gently pull back on the hind limb of the rat so that the dorsal surface of the paw is on the testing surface; release the hind limb)

- 1 = Returns leg to original position
- 2 = Returns leg only partially to original position
- 3 = No response, rat allows leg to remain in pulled back position

Pain perception (tail pinch; using forceps, lightly pinch the animal's tail approximately 2 in. from the tip)

- 1 = Normal; turns or walks forward, away from the stimulus
- 2 = No reaction
- 3 = Exaggerated response; jumps forward, with or without vocalization, or exhibits highly exaggerated, bizarre reaction, attacks, bites

Surface or air righting reflex—The animal is held with its dorsal surface flat on the surface of the lab bench or 12 in. above the surface and is then released. The animal's ability to right itself, such that all four feet are in contact with the surface, is graded as follows:

- 1 = Normal; immediate righting
- 2 = Slow or poorly coordinated righting
- 3 = Very slow or fails to achieve righting within approximately 5 s

- **Forelimb and hind limb grip strength**—Hold the animal with its forepaws resting within, but not touching the triangular pull bar of the grip strength meter. Grasp the animal by the base of its tail and slowly pull it across the bar. As the animal's forepaws grip the triangular pull bar, its grip strength will register on the display. Continue pulling the animal after it has released the triangular pull bar. The animal's hind limbs will then contact, and grip, the T-shaped push bar. The hind limb grip strength will register on the display. Repeat the measurements.
- **Landing foot splay measurements**—Grasp the animal by the scruff of the neck and the base of the tail. Hold the animal above the sand at a height of 1 ft. Release the animal; measure the distance between the marks left by the animal's hind paws, measuring from the approximate middle of the animal's heel marks (or record the distance between ink or paint spots). Record the foot splay distance and repeat the test. [Note: An obviously impaired animal or one of questionable viability should be excluded from this evaluation.] Return the animal to its cage.
- **Body temperature**—Record rectal temperature using digital electronic thermometer.

TABLE 7.10
Chemicals Commonly Used as Positive Control Materials
for the FOB

Chemical	Effect
Acrylamide	Increased landing foot splay, decreased grip strength (especially hind limb), ataxia, decreased motor activity, tremors
Amphetamine	Increased arousal, increased locomotion, increased rearing, stereotypical behavior
Carbaryl } Physostigmine } Parathion }	Autonomic signs (salivation, lacrimation, miosis), tremor, muscle fasciculations, altered gait, decreased activity, hypothermia, chewing motions
Chlorpromazine	Decreased activity, low arousal, flattened posture, altered gait, decreased grip strength
Dichlorodiphenyltrichloroethane (DDT)	Tremors, myoclonus, convulsions, increased response to auditory stimuli, gait abnormalities, hyperthermia
Triethyltin	Altered gait, decreased grip strength, decreased righting ability, decreased activity and arousal

Source: Adapted from Moser, V.C. and Ross, J.F., U.S. Environmental Protection Agency and American Industrial Health Council, 1996.

TABLE 7.11
Measures of the FOB, Divided by Functional Domain

Functional Domain	FOB Measures
Activity	Home cage posture, palpebral closure, locomotion and rearing in open field, automated measure of motor activity
Autonomic	Lacrimation, salivation, pupil response, palpebral closure, defecation, urination
Convulsive	Tonic and clonic movements, tremors, myoclonus
Excitability	Arousal, removal and handling reactivity, vocalizations
Neuromuscular	Gait, locomotion, forelimb and hind limb grip strength, landing foot splay, air righting
Sensorimotor	Tail-pinch response, auditory or click response, touch response, approach response
Physiological measures	Body weight, body temperature, piloerection

Source: Adapted from Moser, V.C., *J. Am. Coll. Toxicol.* 10, 661, 1991.

TABLE 7.12
Profile of FOB Effects across Functional Domains for Known Neurotoxicants

Functional Domain	FOB Measure Affected		
	Acrylamide ^a	p,p'-DDT ^b	Parathion ^c
Activity	Decreased motor activity, some decreased rearing, flattened posture in home cage	No consistent effects	Decreased motor activity, decreased rearing, flattened posture in home cage
Autonomic	No consistent effects	No consistent effects	Salivation, miosis
Convulsive	Some tremors, myoclonus	Tremors (mild to severe), some clonic convulsions, myoclonus	Tremors, chewing
Excitability	No consistent effects	Some increased reactivity to removal from home cage, increased handling reactivity	Decreased arousal level
Neuromuscular	Altered gait (ataxia, uncoordinated placement of hind limbs, splaying of hind feet, knuckling of paws, dragging the hind limbs), increased landing foot splay, decreased hind limb and forelimb grip strength	Altered gait (ataxia, uncoordinated placement of limbs)	Altered gait (ataxia) and righting reflex, some decreases in forelimb and hind limb grip strength
Sensorimotor	No consistent effects	Increased click response, increased touch response	Decreased response to tail pinch, some decreases in click and touch response
Physiological measures	Decreased weight gain, hypothermia	Hyperthermia	Marked hypothermia

Source: Based on data from Moser, M.V. et al., *Neurotoxicology*, 18, 969, 1997.

Note: Results were compiled from a collaborative study involving several laboratories. The findings presented are the significant observations generally found for the chemicals by the participating laboratories.

^a Major effects observed after 4 weeks of dosing at 28.8 mg/kg via intraperitoneal injection.

^b Acute effects observed 1–6 h after oral dose ranging from 58.0 to 195.8 mg/kg.

^c Acute effects observed 1.5–3 h after oral dose ranging from 3.0 to 10.13 mg/kg.

MOTOR ACTIVITY

Monitoring motor activity via an automated activity recording device is an integral part of neurobehavioral assessment. The type of device is not specified by the regulatory agencies, although basic criteria for its capabilities and use must be met.

1. The device must be capable of detecting increases and decreases in activity, that is, baseline activity as measured by the device must not be so low as to preclude detection of decreases or so high as to preclude detection of increases in activity. Positive control data demonstrating that this criterion has been met can be generated by treating animals with chemicals such as amphetamine or triadimefon to produce an increase in activity or chlorpromazine to produce a decrease in activity.
2. Each animal should be tested individually. When an animal is initially placed into a unit of the motor activity device, it generally displays high activity as it explores the new environment. As the animal becomes acclimated during the test session, activity drops off asymptotically. To evaluate whether an animal displays this normal pattern of activity, the test session should be long enough for motor activity to approach asymptotic levels by the last 20% of the session for nontreated control animals.

3. Variations in environmental conditions (e.g., lighting, sound, activity) across treatment groups must be minimized.
4. Treatment groups must be counterbalanced across motor activity devices if multiple devices are used during a test session. Treatment groups must also be counterbalanced across the time of day.

The most common devices for measuring motor activity include those that use photodetectors and video imaging. Video imaging devices measure the total distance traveled or the number of squares crossed in a set time interval. Photodetectors measure the number of beam breaks during the test session. The number of photobeams varies as does the shape of the individual motor activity units (square, rectangular, and figure-8 are the most common).

Typical motor activity data for male Sprague–Dawley rats are presented in Figure 7.1. Motor activity was monitored using a Kinder Scientific Motor Monitor 7 × 15 High Density Cage Rack System, version 6.00. The system consists of rectangular plastic shoe-box cages, each of which is surrounded by a frame embedded with photobeams in both the horizontal and vertical planes. Each test session was 60 min in length; each session was divided into 12 5-min intervals. Figure 7.1a and b show the increases and decreases in motor activity in the horizontal and vertical planes, respectively, after treatment with amphetamine or chlorpromazine.

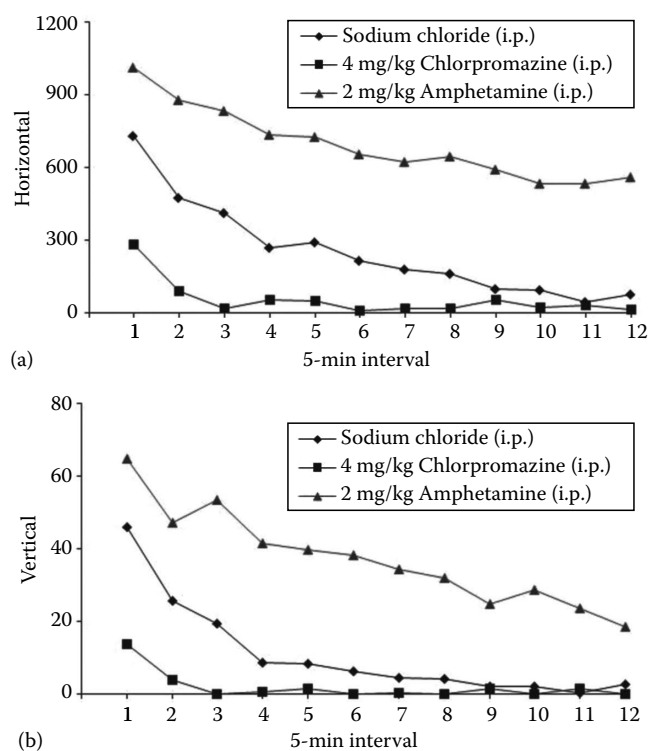


FIGURE 7.1 (a) Mean motor activity values (number of beam breaks) in the horizontal plane for male Sprague–Dawley rats treated with saline (control), amphetamine, or chlorpromazine. (b) Mean motor activity values (number of beam breaks) in the vertical plane for male Sprague–Dawley rats treated with saline (control), amphetamine, or chlorpromazine.

NEUROLOGICAL EVALUATIONS IN DOGS AND NONHUMAN PRIMATES

Dogs and monkeys are generally not used for neurotoxicity screening studies. The reasons for this center on questions of the appropriate use of animals in toxicity studies, as well as practical considerations. The cost and availability of these animals limit the number of animals that can be placed on study, thereby affecting the potential for detecting a neurotoxic effect. Cost and availability, as well as animal welfare issues, would also generally preclude the use of dogs and monkeys in validation studies with a known neurotoxicant. In addition, some of the manipulative tests that are done with rodents are not easily done with the larger animals. This is especially true for nonhuman primates since handling monkeys can easily affect their behavior and may also pose a safety problem for the observer. The genetic background and breeding of rodents are also much more standardized

than that of dogs and monkeys. Differences in the sources of the larger animals, housing conditions, socialization, interaction with humans, and ages can all have an effect on their neurological status.

However, under certain conditions it may be useful or necessary to evaluate systematically the neurological condition of a dog or a monkey. This may occur, for example, during studies with pharmaceuticals that are targeted at the nervous system or when possible neurological effects are observed during general physical examinations and a more detailed evaluation is needed to describe the effect fully. Evaluation schemes for dogs and monkeys are presented in Tables 7.13 and 7.14, respectively. As with the rodent studies, the possibility for bias is of concern, and factors such as environmental conditions should be controlled. Because of the smaller number of dogs and monkeys in a study and the individual variability in appearance that is inherent in the larger animals, performing “blinded” observations is much more difficult.

TABLE 7.13
Neurological Evaluation in the Dog

Behavior in Home Cage

Alertness

1. Normal, active (aware of surroundings, responds to the presence of observer by moving or jumping around cage)
2. Normal, quiet (aware of surroundings, approaches observer)
3. Agitated (appears hypersensitive to stimuli, may respond aggressively to observer)
4. Low arousal (unresponsive to presence of observer, responds to physical stimuli)
5. Very low arousal (unresponsive to presence of observer or to physical stimuli)
7. Coma, unconsciousness

Position

1. Normal sitting or standing position
2. Sitting in a hunched position with head down
3. Lying down, ventrally recumbent
4. Lying on side
5. Lying down with limbs splayed

Position of eyelids

1. Eyes fully open
2. Eyelids half closed
3. Eyelids completely closed

Salivation

1. Present (note severity)
2. Not present

Lacrimation

3. Present (note severity)
4. Not present

Piloerection

5. Present
6. Not present

Abnormal motor movements (describe, e.g., tremors, convulsions, stereotypy, indicate location and severity)

1. Present
2. Not present

Out of Cage Evaluations

Observe the dog and note any abnormalities of gait (e.g., ataxia, staggering), locomotion (decreased or no movement), position of the head and neck, torso, and tail, symmetry of facial muscles, position of the eyes. Check muscle tone (e.g., normal, rigid, flaccid) while handling the dog.

Reflex Activity

For evaluation of reflex activity, record any responses that are not exactly as expected (e.g., decreased but not absent).

Pupillary reflex—Shine a penlight beam into the animal's eyes, one at a time. Hold the light beam for approximately 5 s and remove the light beam from the animal's eyes. The expected reaction is pupil constriction with the light and pupil dilation when the light is removed. Score as follows:

- 1 = Present
- 2 = Not present

Patellar reflex—Lift one of the animal's hind legs, preventing the animal from placing any weight on the leg. Hold the leg loosely and tap the knee with a percussion hammer. The expected reaction is for the animal to kick its leg slightly in response to the tap. Score as follows:

- 1 = Present
- 2 = Not present

Flexor reflex—Firmly grasp one of the animal's forepaws and slowly pull forward. The expected reaction is for the animal to pull its leg back. Score as follows:

- 1 = Present
- 2 = Not present

Extensor reflex—One of the animal's forepaws is placed in the palm of the examiner's hands and the forepaw is pushed toward the dog. The expected response is for the animal to push against the hand. Score as follows:

- 1 = Present
- 2 = Not present

(continued)

TABLE 7.13 (continued)
Neurological Evaluation in the Dog

Corneal reflex—The examiner holds a hand directly in front of and approximately 1 ft from the animal's face. The examiner moves his hand quickly toward the animal's face. The expected response is for the animal to blink its eyes.

Postural Reactions

Righting ability—Place the animal on its right side and observe its reactions. Repeat with the left side. The expected reaction is for the animal to right itself. Score as follows:

- 1 = Able to achieve sternal recumbency or stand easily
- 2 = Able to achieve sternal recumbency or stand only with unusual effort
- 3 = Unable to achieve sternal recumbency or standing position

Visual placing response—Hold the animal around its midsection with its forelimbs free and facing away from the holder. Walk slowly toward a flat surface (table, laboratory cart) and observe the animal's ability to reach with its forelimbs to the surface (the expected response). Score as follows:

- 1 = Reaches for surface with forelimbs
- 2 = Does not reach for surface

Tactile placing response—Same procedure as visual placing response, except that the animal's eyes are covered. The animal is moved toward the surface until its legs bump gently against the edge. Observe ability to extend its legs toward the table in response to the tactile stimulus (expected response). Score as follows:

- 1 = Reaches for the surface with forelimbs
- 2 = Does not reach for the surface

Proprioceptive positioning—While the animal is standing on all four feet, each foot is picked up in turn and replaced on the floor in a "knuckled over" position. The ability to correctly reposition the foot is noted. Score as follows:

- 1 = Quickly and easily repositions feet
- 2 = Difficulty in repositioning feet is noted (record which feet affected)
- 3 = Cannot reposition feet correctly

Extensor postural thrust—Pick up the animal and hold it such that the hind feet dangle below it. Slowly lower the dog to the floor and note how readily and capably it bears weight on its hind legs. Move the animal from side to side and notice how well it keeps up with its hind feet. Score as follows:

- 1 = Easily bears weight, and movements coordinated with examiner
- 2 = Easily bears weight, but movements are not coordinated with examiner
- 3 = Difficulty in bearing weight

Wheelbarrowing—Pick up the dog's hind legs and move the animal about the room. Note its ability to bear weight on its front legs and its coordination in moving right and left legs. Score as follows:

- 1 = Easily bears weight and has coordinated movement
- 2 = Easily bears weight, but movements are uncoordinated
- 3 = Difficulty in bearing weight

Hemistands/hemiwalks—Pick up the front and hind legs on the left side and observe ability to bear weight on the right side. Prompt the animal to move on the front and hind right side and observe functional ability. Repeat for the left side. Score for each side as follows:

- 1 = Easily bears weight and movements are coordinated
- 2 = Easily bears weight but moves with difficulty
- 3 = Difficulty in bearing weight

TABLE 7.14
Neurological Evaluation of Nonhuman Primates

Behavior in Home Cage

Observe each animal in its home cage. Eye contact with the animal causes stress and should be avoided.

Alertness

- 1. Normal, active (directs attention to the presence of the observer, moves energetically around cage, shows normal aggressive behavior, e.g., showing teeth)
- 2. Normal, quiet (directs attention to the presence of the observer, moves calmly around cage, but lacks normal aggressive behavior)
- 3. Low arousal state (little or no activity, does not respond to presence of the observer, responds to physical stimuli)
- 4. Very low arousal state (unconscious, no response to the observer or physical stimuli)

Posture

- 1. Normal—sitting or standing on a perch or cage bars
- 2. Sitting in a hunched position
- 3. Lying down

TABLE 7.14 (continued)
Neurological Evaluation of Nonhuman Primates

Lacrimation

1. Not present
2. Present
3. Excessive

Salivation

1. Not present
2. Present
3. Excessive

Observe the animal for any of the following abnormal motor movements. Describe fully with regard to type, location, and severity:

- Tremors
- Convulsions—specify clonic or tonic
- Fasciculations
- Stereotypic behavior (e.g., circling, continuous rhythmic movements, compulsive grooming)

Cranial Nerve Function

Note any abnormal head positions (e.g., head tilt)

Movement of the facial muscles

1. Symmetrical
2. Asymmetrical

Position of the eyelids

1. Eyelids open
2. Eyelids slightly drooping
3. Eyelids half closed
4. Eyelids completely closed

Pupillary light response—Shine a penlight beam into the animal's eyes, one at a time:

1. Pupils constrict
2. No response

Visual field—Hold an object in front of the monkey and move it laterally and up and down. Observe the ability of the monkey to follow the movements:

1. Follows all movements symmetrically
2. Asymmetry noted in following movements
3. Cannot move eyes laterally
4. Cannot move eyes up and down

Auditory response—Note the animal's response to a sudden sound produced by a "clicker" or other noisemaker:

1. Responds to noise
2. No response

Response to food—Present a small food treat to the animal (e.g., raisin, grape) and observe the ability of the animal to chew and swallow the treat.

1. Movements smooth and symmetrical
2. Difficulty with movements/not symmetrical
3. Unable to chew or swallow
4. No response to food

Motor Function

Observe the motor ability of the primate while it is involved in various activities, example, ability to pick up a treat and place it in its mouth and ability to use limbs in climbing about the cage. Strength can be evaluated by presenting an examination probe to the animal and noting how firmly it grasps the probe. Any abnormalities should be noted with an indication of affected extremity.

1. Normal function and strength (normal use of extremities, no apparent differences between right and left sides, firmly grasps examination probe)
2. Normal strength, but appears to favor one side (note any physical reasons for this, e.g., sore on one foot)
3. Paresis (partial or incomplete deficit of motor function, affected extremity may be used for some functions, but is unable to grasp examination probe firmly; note location of deficit and severity)
4. Loss of voluntary movement (affected extremity not functional and animal is unable to grasp examination probe)

NEUROPATHOLOGY

Alterations in any of the structures comprising the nervous system are considered evidence of a neurotoxic effect. It is important to realize in evaluating structural effects of a compound that, in many cases, there is a lag time between exposure to a neurotoxicant and the ability to observe the lesion microscopically. Another factor to consider in microscopic evaluations is the potential for producing histological artifacts during the handling and processing of neuronal tissues. Neuronal tissue is somewhat delicate because of the lack of connective tissue and the high lipid content, and requires a great deal of care to visualize details at the microscopic level. Thus, regulated neurotoxicity studies generally include *in situ* tissue fixation by whole-body perfusion and the use of an aldehyde fixative (generally a combination of paraformaldehyde and glutaraldehyde).

In consideration of the diversity of the nervous system and the local specificity of action of neurotoxicants, it is essential that samples of the nervous system should be representative of all the major regions of the nervous system. A list of the areas generally sampled is found in Table 7.15, and typical neuropathologic effects in the CNS and PNS of rats is given in Table 7.16.³² It is considered acceptable to embed the brain and spinal cord in paraffin, but it is recommended that peripheral nerves be embedded in plastic for better observation of the details in those structures. General stains such as hematoxylin and eosin for paraffin-embedded tissues and toluidine blue for plastic-embedded tissues are standard, but additional stains are recommended to investigate further any observed changes in the nervous system. A list of some specialized stains is included in Table 7.17.³³

TABLE 7.15

Representative Areas of the Nervous System for Histopathological Evaluations

Brain

Section 1 (coronal incision rostral to olfactory tubercles): cerebral cortex, rhinal fissure, olfactory tracts

Section 2 (coronal incision through optic chiasm): cerebral cortex, corpus callosum, basal ganglia (globus pallidus, putamen, caudate nucleus), thalamus, hypothalamus, internal capsule, external capsule, lateral ventricles, third ventricle

Section 3 (coronal incision through infundibulum): cerebral cortex, corpus callosum, hippocampus, amygdala, thalamus, hypothalamus, lateral ventricles, third ventricle, internal capsule, external capsule

Section 4 (coronal incision at caudal margin of the mammillary body): cerebral cortex, hippocampus, medial geniculate nuclei, substantia nigra, cerebral aqueduct

Section 5 (coronal incision at caudal border of the trapezoid body): cerebellum, cerebellar peduncles, pyramidal tract, medulla, fourth ventricle

Section 6 (coronal incision through medulla immediately beneath the caudal edge of the cerebellum): medulla, pyramidal tract, olivary nuclei, central canal

Trigeminal ganglia

Eye, with optic nerve and retina

Spinal cord

Cervical (longitudinal and cross sections)—at cervical enlargement

Thoracic (longitudinal and cross sections)

Lumbar (longitudinal and cross sections)—at lumbar enlargement

Dorsal root ganglia and associated dorsal and ventral root fibers

Cervical region

Lumbar region

Peripheral nerves

Sciatic nerve (proximal region)

Tibial nerve (proximal, at the knee)

Tibial nerve and calf muscle (distal, at calf muscle)

Sural nerve

TABLE 7.16
Prototypic Neurotoxic Pathology in the CNS and Peripheral Neuromuscular System of the Rat

Anatomical Site	Axonopathy	Myelinopathy	Neuronopathy
CNS			
Cortical neurons ^b	0	0	±
Ventral hypothalamus ^a	0	0	++e
Subfornical organ	0	0	+e
Area postrema	0	0	+e
Lateral geniculate body	++d	±	±
Optic tract	+d	±	±
Optic nerve	0	±	±
Retina	0	±	±
Cerebellar vermis	++d	±	±
Gracile nucleus ^{a,c,d}	++d	0	++s
Cuneate nucleus ^a	0	0	++s
Gracile tract (T6)	0	0	++s
Gracile tract (L5)	0	0	++s
Ventromedial tract (medulla oblongata) ^a	0	+	0
Ventromedial tract (T6)	+d	+	0
Ventromedial tract (L5)	++d	+	0
Dorsal spinocerebellar tract (medulla oblongata)	+d	+	0
Hypoglossal nucleus ^b	0	0	±
Descending tract of V	0	0	++s
Lumbar cord, anterior horn ^b	+p	0	+m
Mammillary bodies ^d	+d	0	0
PNS			
Gasserian ganglion ^b	0		++s
Lumbar dorsal root ganglia ^b	0	±	++s
Lumbar dorsal root	0	±	++s
Lumbar ventral root ^c	0	0	+m
Proximal sciatic nerve	±d	+	+
Tibial nerve at knee ^c	+d	+	+
Tibial calf muscle branches ^f	++d	++	++
Plantar nerves at ankle ^c	+d	+	+
Sural nerve at knee ^g	±d	+	++s
Gastrocnemius muscle ^h	+d	±	±
Lumbrical muscle spindles ⁱ	+d	0	+s
Lumbar neuromuscular junctions ^h	+d	0	0

Source: Spencer, P.S. and Schaumburg, H.H., *Experimental and Clinical Neurotoxicology*, Williams & Wilkins, Baltimore, MD, 1980, Chapter 50. With permission.

Notes: ++, Great vulnerability; +, less or late (for distal axonopathy) vulnerability; ±, variable or very late (for distal axonopathy) vulnerability; 0, no or little vulnerability; e, excitotoxin; s, sensory neuronopathy only; m, motor neuronopathy only; p, proximal axonopathy only; d, distal axonopathy only.

^a Used to distinguish the three types of neurotoxic disease.

^b Amount of neuronal lipofuscin increases with age.

^c Aged animals display axonal changes in gracile nucleus (6 mo plus), along with scattered myelin bubbles and remyelination in ventral root (1–2 yr plus).

^d Neuronal degeneration may be seen in normal animals.

^e Plantar nerves are vulnerable to nerve entrapment; changes increase with age (6 mo plus) and may spread to involve tibial nerve trunk.

^f Best locus to prepare teased nerve fibers.

^g Composed predominantly of sensory nerve fibers.

^h Located on extrafusal muscle fibers only.

ⁱ Sensory innervation in mideduatorial zone.

TABLE 7.17
Specialized Stains Used in Neurotoxicity Evaluations

Structure	Stain	Appearance
Nissl substance	Thionin stain	Nerve cells—bright blue Background—colorless
	Cresyl echt Violet for Nissl substance	Nissl substance—blue
Nerve fibers (axons and dendrites) and intracellular neurofibrils	Bielschowsky's method for neurofibrils (silver stain)	Intracellular neurofibrils—black
	Bodian's method (silver stain)	Myelinated fibers, the finest nonmyelinated fibers of CNS and PNS—black
	Sevier–Munger modification of Bielschowsky's method (silver stain)	Nerve endings and neurofibrils—black Other elements—light brown
Neuroglia	Toluidine blue	Nerve cells Nucleus—pale blue Nissl bodies—dark blue Glia Astrocytes—pale blue Oligodendroglia—very dark blue
	Cresyl echt Violet stain for nerve cells and glia	Nerve cells Nucleus—pale blue Nissl bodies—dark blue Glia Astrocytes—pale blue Oligodendroglia—very dark blue
	Holzer's stain for glia fibers	Glia fibers—blue
Myelin	Luxol fast blue method	Myelin fibers—blue to greenish blue Cells—pink to violet
	Luxol fast blue-periodic acid schiff-hematoxylin	Myelin sheath—blue green Capillary basement membranes—rose Nuclei—purple
	Luxol fast blue—Holmes' silver nitrate	Myelin sheath—blue to green Nerve fibers—black
	Luxol fast blue—Phosphotungstic acid hematoxylin	Myelin—blue Glial fibers—purple
	Luxol fast blue—oil red O	Fat—red Myelin sheath—blue
Degenerated myelin	Marchi's method	Degenerating myelin—black Background—brown/yellow

Source: Carson, F., Nerve tissue, in *Theory and Practice of Histotechnology*, Sheehan, D.C. and Hrapchak, B.B., Eds., Battelle Press, Columbus, OH, 1980, Chapter 14. With permission.

NEUROCHEMICAL AND ELECTROPHYSIOLOGICAL MANIFESTATIONS OF NEUROTOXICITY

Many different neurochemicals have been identified and measured in tissues of the nervous system. These include neurotransmitters, receptors, second messengers, and metabolic and catabolic enzymes. The presence of a change in a particular neurochemical parameter is not per se an indication of a toxic effect, since many neuroactive compounds can cause pharmacological changes. If, however, the neurochemical changes are correlated with neurophysiological, neuropathological, or neurobehavioral effects, then the neurochemical changes may be classified as neurotoxic effects. In most toxicity studies, neurochemical changes have been investigated to enhance the understanding of the mechanism of neurotoxic action, rather than to predict the neurotoxic potential of a particular agent. There are only a few cases in which neurochemical end points are used to screen for neurotoxicants

and are included in neurotoxicity study guidelines. These end points are listed in Table 7.18.^{34–37} In addition to those parameters, a number of chemicals specific for neurons and glia are under investigation for possible use in predicting neurotoxic effects. Table 7.19^{35,39–43} lists some of the neurotypic and gliotypic proteins for which alterations in levels have been associated with the actions of known neurotoxicants.

One distinguishing property of nervous tissue is its ability to generate and propagate electrical signals. This property is probably its most important physiological function. In this regard, there are several ways to measure the electrical properties of the nervous system. These techniques may be used in various species and generally are noninvasive.³⁸ The use of these tests is also prescribed in the regulatory guidelines for assessing neurotoxicity. Table 7.20⁴⁴ lists various noninvasive electrophysiological tests that can be used to detect and characterize neurotoxicity.

TABLE 7.18
Neurochemical End Points Used in Neurotoxicity Screening

Marker of Neurotoxicity	Localization	Neurotoxic Effects	Examples of Neurotoxicants	References
Glial fibrillary acidic protein (GFAB)	Astrocytes	Astrocytes proliferate and hypertrophy in response to damage to the nervous system (reactive gliosis), resulting in an accumulation of GFAP; GFAP can be localized by immunocytochemistry and quantitated by immunoassays.	MPTP Trimethyltin Triethyltin Tributyltin Methylmercury Domoic acid Bilirubin Kainate IDPN Methamphetamine	[34,35]
Acetylcholinesterase activity	Synapses of cholinergic neurons in CNS and PNS	Acetylcholinesterase hydrolyzes the neurotransmitter acetylcholine; inhibition of enzyme activity prolongs the action of acetylcholine at the neuron synaptic receptors, resulting in exaggerated cholinergic effects; measurement of plasma and erythrocyte activity serves as a biomarker for peripheral effects.	Organophosphate (OP) insecticides (e.g., parathion, malathion, and chlorpyrifos) Carbamate insecticides (e.g., aldicarb, carbaryl, carbofuran)	[36]
Neuropathy target esterase (NTE) or neurotoxic esterase	Neuronal membrane-bound enzyme	The enzyme hydrolyzes phenyl valerate, although its cellular function is not known; the inhibition and “aging” of the phosphorylated NTE, that is, the covalent binding of an organophosphate to the enzyme, is highly correlated with the initiation of organophosphate-induced delayed neurotoxicity (OPIDN); the hen is more sensitive than rodents and used in regulatory testing.	OP compounds (e.g., mipafox, leptophos, methamidophos, trichlorphon, and chlorpyrifos), not all OPs that inhibit NTE cause OPIDN, but all OPs that cause OPIDN inhibit NTE	[37]

TABLE 7.19**Examples of Neurotypic and Gliotypic Proteins Altered by Neurotoxics**

Protein	Localization	Neurotoxicant	Area Affected	References
Synapsin I	Synaptic vesicles, all CNS and PNS neurons	Bilirubin	Cerebellum	[35,39]
		Trimethyltin	Hippocampus	
Protein III	Synaptic vesicles, all CNS and PNS neurons	Trimethyltin	Hippocampus	[40]
p38	Synaptic vesicles, all CNS and PNS neurons	Trimethyltin	Hippocampus	[39]
Purkinje cell-specific phosphoprotein (PCPP-260)	Cerebellar Purkinje cells	Bilirubin	Cerebellum	[35]
P68	Axonal intermediate filaments, all CNS and PNS neurons	Trimethyltin	Hippocampus	[39]
Protein-O-carboxymethyltransferase (PCM)	Cytosol, CNS neurons	Trimethyltin	Hippocampus	[41]
Neuron-specific enolase (NSE)	Cytosol, CNS and PNS neurons	Trimethyltin	Hippocampus	[39,42]
		<i>n</i> -Hexane	Distal sciatic nerve	
		Toluene	Cerebellum	
Creatine kinase-B	Cytosol, CNS neurons	<i>n</i> -Hexane	Distal sciatic nerve	[42]
		Toluene	Cerebellum	
Myelin basic protein	Myelin, oligodendrocytes	Bilirubin	Cerebellum	[35,43]
		Trimethyltin	Hippocampus	

TABLE 7.20**Noninvasive Electrophysiological Tests for Neurotoxicity Testing**

Peripheral	Needle electromyography Single/repetitive evoked muscle potentials Sensory/mixed nerve evoked potentials F-Wave H-Wave
Central	Spontaneous electroencephalogram Visual evoked potential Pattern reversal evoked potential Brainstem auditory evoked response Somatosensory evoked response
Cardiac	Electrocardiogram

Source: Ross, J.F., *Toxicol. Ind. Health*, 5, 221, 1989. With permission.

MOLECULAR TARGETS OF NEUROTOXICANTS

At the molecular level, neurotoxics may act on nervous tissues by interacting with a variety of biochemical processes.^{45–48} The most important property of nervous tissue is its ability to transmit information. This is accomplished by the propagation of electrical or chemical signals. These processes are vulnerable to disruption by chemicals. Electrical signals in the neuron are propagated by the movement of ions across the membrane through various ion-selective channels. These channels can often act as the focus of neurotoxicant action.⁴⁷ Chemical signals are propagated by the presynaptic release of neurotransmitters that diffuse across intercellular spaces and interact

with receptors on adjacent cell membranes. These neurotransmitters, receptors, and associated second messengers, anabolic, and catabolic processes may also be the target for neurotoxicant action.^{45,47,48} In addition, nervous tissue has a high metabolic demand and, because of its energy requirement, nervous tissue is extremely sensitive to compounds that interfere with energy metabolism.⁴⁹ Tables 7.21⁴⁷ and 7.22 provide examples of neurotoxins that interact with ion channels, cellular biochemistry, and neurotransmitter systems. Table 7.23⁵⁰ provides information regarding methods for characterizing neurotransmitter receptor binding sites that are used in neurotoxicity evaluations.

TABLE 7.21
Examples of Chemicals That Produce Their Effect
by Acting on Neuronal Ion Channels

Ion Channel	Blockers	Modulators
Voltage-Activated Channels		
Sodium	Tetrodotoxin	Batrachotoxin
	Saxitoxin	Grayanotoxin
	Local anesthetics	Veratridine
	Pancuronium	Pyrethroids
	<i>N</i> -Alkylguanidines	DDT
		Goniopora toxin
		Sea anemone toxins
		Scorpion toxins
		Pronase
		<i>N</i> -Bromoacetamide
Potassium	Tetraethylammonium	
	Aminopyridines	
	Cesium	
	Local anesthetics	
Calcium	Dihydropyridines	Bay K8644
	Diltiazem	
	Verapamil	
	Enkephalins	
	Phenytoin	
	Polyvalent cations	
Chemically Activated Channels		
Acetylcholine receptor	Local anesthetics	
	Histronicotoxin	
	Amantadine	
	<i>N</i> -Alkylguanidines	
L-Glutamate receptor	Magnesium	
	2-Amino-5-phosphonovaleric acid (2-APV)	
	γ -D-glutamylglycine	
	Barbiturates	
	Bicuculline	Baclofen
GABA receptor	Picrotoxin	Barbiturates
	Saclofen	Benzodiazepines
		Muscimol

Source: Narahashi, T., Cellular electrophysiology, in *Neurotoxicology*, Abnou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 169. With permission.

TABLE 7.22
Examples of Subcellular Targets for Neurotoxin Action

Subcellular Target	Toxin	Biochemical Target
Nucleus	Actinomycin D	DNA replication
Ribosome	Cycloheximide	Protein synthesis
	Emetine	
Rough endoplasmic reticulum	Tunicamycin	Glycosylation
Mitochondria	Cyanide	Electron transport
Axon	<i>n</i> -Hexane	Axoplasmic transport
	Colchicine	Microtubules
	Cytochalasin	Neurofilaments
	Tetrodotoxin	Sodium channels
	TEA	Potassium channels
Presynaptic end plate	Hemicholinium	Choline uptake
	Botulinum toxin	Acetylcholine release
Synaptic cleft	Physostigmine	Acetylcholine esterase
Postsynaptic end plate	Bungarotoxin	Acetylcholine receptor

TABLE 7.23
Agents Used to Characterize Neurotransmitter
Receptor Binding

Labeled Ligand	Unlabeled Competitor	Neurotransmitter Receptor
DL-[Benzilic-4,4'- ³ H] ⁴	Atropine	Muscarinic cholinergic
Quinuclidinyl benzilate		
[1-Phenyl-4- ³ H]Spiroperidol	(+)Butaclamol	Dopamine
[Methylene- ³ H(N)]Muscimol	GABA	GABA
[Methyl- ³ H]Diazepam	Diazepam	Benzodiazepine
[1,2- ³ H(N)]Serotonin	Serotonin	Serotonin
[G- ³ H]Strychnine sulfate	Strychnine	Glycine
9-10-[9,10- ³ H(N)]	Ergocryptine	α -Adrenergic
Dihydro- α -ergocryptine		
Levo-[propyl-1,2,3- ³ H]	Alprenolol	β -Adrenergic
Dihydroalprenolol		
[<i>N</i> -allyl-2,3- ³ H]Naloxone	Levallorphan	Opiate
Opiate Subtypes		
[tyrosyl-3,4- ³ H(N)]DAGO	Etorphine	μ
[9- ³ H(N)]Bremazocine	Etorphine	κ
[tyrosyl-3,5- ³ H(N)]Enkephalin	Etorphine	δ
[piperidyl-3,4- ³ H(N)]TCP	Phencyclidine	σ (PCP)

Source: Bondy, S.C. and Ali, S.F., Neurotransmitter receptors, in *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 129. With permission.

INTERNET WEB SITES RELATED TO NEUROTOXICITY

braininfo.rprc.washington.edu

Portal to neuroanatomical information on the web. Includes atlas of nonhuman primate brains.

mbl.org

Mouse brain library, includes brain sections for over a 100 mouse strains.

neuroguide.com

A searchable index of neuroscience resources available on the Internet.

Vanat.cvm.umn.edu

Gross and microscopic anatomy of the canine brain.

REFERENCES

- Goyer, R.A., Toxic effects of metals, in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., Ed., McGraw-Hill, New York, 1996, p. 722.
- Lukiw, W.J. and McLachlan, D.R., Aluminum neurotoxicity, in *Handbook of Neurotoxicity*, Chang, L.W. and Dyer, R.S., Eds., Marcel Dekker, New York, 1995, Chapter 4.
- Norton, S., Toxic effect of plants, in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., Ed., McGraw-Hill, New York, 1996, p. 846.
- Tryphonas, L., Trulove, J., Nera, E., and Iverson, F., Acute neurotoxicity of domoic acid in the rat, *Toxicol. Pathol.*, 18, 1, 1990.
- Tryphonas, L., Trulove, J., and Iverson, F., Acute parenteral neurotoxicity of domoic acid in cynomolgus monkeys, *Toxicol. Pathol.*, 18, 297, 1990.
- Strain, S.M. and Tasker, R.A., Hippocampal damage produced by systemic injection of domoic acid in mice, *Neuroscience*, 44, 343, 1991.
- Anthony, D.C., Montine, T.J., and Graham, D.G., Toxic responses of the nervous system, in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C. D., Ed., McGraw-Hill, New York, 1996, p. 466.
- Chu, N.-S., Hochberg, F.H., Calne, D.B., and Olanow, C.W., Neurotoxicity of manganese, in *Handbook of Neurotoxicity*, Chang, L.W. and Dyer, R.S., Eds., Marcel Dekker, New York, 1995, Chapter 3.
- Abou-Donia, M.B., Metals, in *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 387.
- Chang, L.W. and Verity, M.A., Mercury neurotoxicity: effects and mechanisms, in *Handbook of Neurotoxicity*, Chang, L.W. and Dyer, R.S., Eds., Marcel Dekker, New York, 1995, Chapter 1.
- Langston, J.W., Ballard, P., Tetrad, J.W., and Irwin, I., Chronic parkinsonism in humans due to a product of meperidine-analog synthesis, *Science*, 219, 979, 1983.
- Burns, R.S., Chiueh, C.C., Markey, S.P., Ebert, M.H., Jacobowitz, D.M., and Kopin, I.J., A primate model of parkinsonism: Selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 4546, 1983.
- Chang, L.W., Neurotoxicology of organotin and organoleads, in *Handbook of Neurotoxicity*, Chang, L.W. and Dyer, R.S., Eds., Marcel Dekker, New York, 1995, Chapter 5.
- Fullerton, P.M. and Barnes, J.M., Peripheral neuropathy in rats produced by acrylamide, *Br. J. Ind. Med.*, 23, 210, 1966.
- Spencer, P.S. and Schaumburg, H.H., Ultrastructural studies of the dying-back process, *J. Neuropathol. Exp. Neurol.*, 36, 300, 1997.
- Graham, D.G., Amarnath, V., Eng, M.A., Kazaks, E.L., Valentine, W.M., and Anthony, D.C., Biomolecular basis for organic solvent neurotoxicity, in *Handbook of Neurotoxicity*, Chang, L.W. and Dyer, R.S., Eds., Marcel Dekker, New York, 1995, Chapter 12.
- Anthony, D.C., Montine, T.J., and Graham, D.G., Toxic responses of the nervous system, in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., Ed., McGraw-Hill, New York, 1996, p. 476.
- Akasaki, Y., Takauchi, S., and Miyoshi, K., Cerebellar degeneration induced by acetyl-ethyl-tetramethyl-tetralin (AETT), *Acta Neuropathol. (Berlin)*, 80, 129, 1990.
- Spencer, P.S., Sterman, A.B., Horoupian, D.S., and Foulds, M.M., Neurotoxic fragrance produces ceroid and myelin disease, *Science*, 204, 633, 1979.
- Love, S., Cuprizone neurotoxicity in the rat: morphologic observations, *J. Neurol. Sci.*, 84, 223, 1988.
- Rose, A.L., Wisniewski, H.M., and Cammer, W., Neurotoxicity of hexachlorophene: new pathological and biochemical observations, *J. Neurol. Sci.*, 24, 425, 1975.
- Tripier, M.F., Berard, M., Toga, M., Martin-Bouyer, G., Le Breton, R., and Garat, J., Hexachlorophene and the central nervous system; toxic effects in mice and baboons, *Acta Neuropathol. (Berlin)*, 53, 65, 1981.
- Anthony, D.C., Montine, T.J., and Graham, D.G., Toxic responses of the nervous system, in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th edn., Klaassen, C.D., Ed., McGraw-Hill, New York, 1996, p. 479.
- Chang, L.W., Neurotoxicology of organotin and organoleads, in *Handbook of Neurotoxicity*, Chang, L.W. and Dyer, R.S., Eds., Marcel Dekker, New York, 1995, Chapter 5.
- McDaniel, K.L. and Moser, V.C., Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin, *Neurotoxicol. Teratol.*, 15, 71, 1993.
- Moser, V.C., McCormick, J.P., Creason, J.P., and MacPhail, R.C., Comparison of chlordimeform and carbaryl using a functional observational battery, *Fundam. Appl. Toxicol.*, 11, 189, 1988.
- O'Donoghue, J.L., Screening for neurotoxicity using a neurologically based examination and neuropathology, *J. Am. Coll. Toxicol.*, 8, 97, 1989.
- Haggerty, G.C., Development of tier I neurobehavioral testing capabilities for incorporation into pivotal rodent safety assessment studies, *J. Am. Coll. Toxicol.*, 8, 53, 1989.
- Moser, V.C. and Ross, J.F., Training video and reference manual for a functional observational battery, U.S. Environmental Protection Agency and American Industrial Health Council, 1996.
- Moser, V.C., Applications of a neurobehavioral screening battery, *J. Am. Coll. Toxicol.*, 10, 661, 1991.
- Moser, V.C., Becking, G.C., Cuomo, V., Frantik, E., Kulig, B.M., MacPhail, R.C., Tilson, H.A. et al., The IPCS collaborative study on neurobehavioral screening methods: V. Results of chemical testing, *Neurotoxicology*, 18, 969, 1997.
- Spencer, P.S. and Schaumburg, H.H., *Experimental and Clinical Neurotoxicology*, Williams & Wilkins, Baltimore, MD, 1980, Chapter 50.

33. Carson, F., Nerve tissue, in *Theory and Practice of Histotechnology*, Sheehan, D.C. and Hrapchak, B.B., Eds., Battelle Press, Columbus, OH, 1980, Chapter 14.
34. O'Callaghan, J.P., Quantitative features of reactive gliosis following toxicant-induced damage of the CNS, *Ann. N.Y. Acad. Sci.*, 679, 195, 1993.
35. O'Callaghan, J.P., Neurotypic and gliotypic proteins as biochemical indicators of neurotoxicity, in *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 71.
36. Costa, L.G., Interactions of neurotoxicants with neurotransmitter systems, *Toxicology*, 49, 359, 1988.
37. Abou-Donia, M.B., Organophosphorus ester-induced delayed neurotoxicity, *Annu. Rev. Pharmacol. Toxicol.*, 21, 511, 1981.
38. Abou-Donia, M.B., Principles and methods of evaluating neurotoxicity, in *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 515.
39. Brock, T.O. and O'Callaghan, J.P., Quantitative changes in the synaptic vesicle proteins synapsin I and p38 and the astrocyte-specific protein glial fibrillary acidic protein are associated with chemical-induced injury to the rat central nervous system, *J. Neurosci.*, 7, 931, 1987.
40. O'Callaghan, J.P. and Miller, D.B., Neuron-specific phosphoproteins as biochemical indicators of neurotoxicity: effects of acute administration of trimethyltin to adult rat, *J. Pharmacol. Exp. Ther.*, 231, 736, 1984.
41. Balaban, C.D., O'Callaghan, J.P., and Billingsley, M.L., Trimethyltin-induced neuronal damage in the rat brain: comparative studies using silver degeneration stains, immunocytochemistry, and immunoassay for neuronotypic and gliotypic proteins, *Neuroscience*, 26, 337, 1988.
42. Huang, J., Kato, K., Shibata, E., Asaeda, N., and Takeuchi, Y., Nerve-specific marker proteins as indicators of organic solvent neurotoxicity, *Environ. Res.*, 63, 82, 1993.
43. Veronesi, B., Pringle, J., and Mezei, C., Myelin basic protein-mRNA used to monitor trimethyltin neurotoxicity in rats, *Toxicol. Appl. Pharmacol.*, 108, 428, 1991.
44. Ross, J.F., Application of electrophysiology in a neurotoxicity battery, *Toxicol. Ind. Health*, 5, 221–230, 1989.
45. Ho, I.K. and Hoskins, B., Biochemical methods in neurotoxicological analysis of neuroregulators and cyclic nucleotides, in *Principles and Methods of Toxicology*, Hayes, A.W., Ed., Academic Press, New York, 1982, pp. 375–402.
46. Miller, M.S. and Spencer, P.S., Single doses of acrylamide reduce anterograde transport velocity, *J. Neurochem.*, 43, 1401–1408, 1984.
47. Narahashi, T., Cellular electrophysiology, in *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 169.
48. Cooper, J.R., Bloom, F.E., and Roth, R.H., *The Biochemical Basis of Neuropharmacology*, 5th edn., Oxford University Press, New York, 1986, p. 359.
49. Norton, S., Toxic response to the central nervous system, in *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Amdur, M.O., Doull, J., and Klaassen, C.D., Eds., Pergamon Press, Elmsford, New York, 1975, pp. 179–205.
50. Bondy, S.C. and Ali, S.F., Neurotransmitter receptors, in *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, p. 129.

8 Immunotoxicology

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INTRODUCTION

The immune system consists of a vast network of lymphoid and nonlymphoid cells, and messenger molecules, such as cytokines and chemokines, that act in an orchestrated manner to protect against infectious and neoplastic diseases. Immunotoxicology is a subdiscipline of toxicology, and the purpose of immunotoxicity testing is to obtain data useful for immunotoxicity safety assessment. Immunotoxicology evaluations are performed to detect immune alterations, stimulatory or suppressive, the immune mechanisms responsible for these alterations, and the resultant effect on susceptibility or duration of infectious, allergic, or autoimmune disease.¹

Immunotoxicology testing began in the mid- to late-1970s as it became evident that exposure to xenobiotics resulted in adverse effects on immunological parameters that were quantifiable.^{2–4} One of the first scientific symposia with a focus on detrimental effects on the immune system was a satellite meeting of the *Annals of the New York Academy of Science* called “Immune Abnormality” in 1979. This meeting was followed by a *Gordon Research Conference on Drug Safety* and the effect of environmental chemicals on the immune system and reported by Dean et al.⁵ In 1983, *Immunology Today* formally announced the birth of the field of immunotoxicology resulting from the fusion of immunology and toxicology.⁶ The formal recognition of immunotoxicology as an important subdiscipline of toxicology coincides with the emergence of acquired immunodeficiency syndrome (AIDS) caused by infection with human immunodeficiency virus (HIV). AIDS emphasized

the severe consequences of immunosuppression and spurred immunotoxicity testing for immunosuppression. It has become increasingly recognized that immunoenhancement, if it induces hypersensitivity or autoimmune disease, can also result in immunotoxicity.

IMMUNOSUPPRESSION

For immunosuppression, the major objective of immunotoxicity testing is to determine the significance of the immunosuppressive effect with respect to increased susceptibility to infectious or neoplastic disease. Thus, immunotoxicity caused by a test compound may be reflected in an impaired clearance of the infectious agent, increased sensitivity to opportunistic infections, prevention of immunization, or exacerbation of latent infections. Measuring the clearance of an infectious microorganism allows an assessment of immunocompetence and serves as a biomarker of net immunological health. Immunosuppression of the immune system may be intended or unintended. Intended immunosuppression may be the result of immunosuppressive drugs, for example, transplant medications or anti-inflammatory drugs such as synthetic glucocorticoid steroids such as dexamethasone (Table 8.1). Other chemicals or pharmaceuticals may cause a direct unintended immunosuppressive effect. Even pharmaceuticals designed to alter the immune system may cause unintended immunotoxicity due to exaggerated pharmacology. In other cases, chemicals or pharmaceuticals may target various lymphoid tissues and cause unintended immunotoxicity as a result of this interaction (Table 8.2).

TABLE 8.1
Examples of Intended Immunosuppression

<i>Prednisone</i> : Glucocorticoid prodrug converted in the liver to the active form, prednisolone; used for suppressing the immune system and as an anti-inflammatory agent; maintenance therapy includes all immunosuppressive medications given before, during, or after transplant with the intention to maintain them long term.
<i>Cyclosporine A</i> : Multiple effect on T cells including binding to the cytosolic protein cyclophilin (immunophilin) of lymphocytes, especially T cells.
<i>Tacrolimus (FK 506)</i> : Macrolide antibiotic; mechanism of action similar to cyclosporine although structurally unrelated; T cell-mediated cytotoxicity is suppressed.
<i>Mycophenolate Mofetil (CellCept®)</i> : Prodrug that is converted to mycophenolic acid (MPA), a potent and reversible noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH); suppresses T-lymphocytic responses to allogeneic cells and other antigens and also suppresses primary, but not secondary, antibody responses.
<i>Azathioprine</i> : Immunosuppressive drug used in organ transplantation and autoimmune diseases; T cells and B cells are particularly affected by the inhibition of purine synthesis.
<i>Rapamycin (Sirolimus)</i> : Prevents activation of T cells and B cells by inhibiting their response to IL-2.
<i>Methylprednisolone</i> : Synthetic glucocorticoid or corticosteroid drug.
<i>Atgam (lymphocyte immune globulin and equine antithymocyte globulin)</i> : Purified, concentrated, and sterile gamma-globulin, consisting primarily of monomeric IgG, from hyperimmune serum of horses immunized with human thymus lymphocytes.
<i>Muromonab-CD3 (trade name Orthoclone OKT3)</i> : A monoclonal antibody directed at the CD3 receptor on the surface of T cells; the first monoclonal antibody approved for clinical use in humans.
<i>Thymoglobulin® (Antithymocyte Globulin [Rabbit])</i> : A purified, sterile, gamma-immune globulin, prepared by immunization of rabbits with human thymocytes; contains cytotoxic antibodies directed against antigens expressed on human T-lymphocytes.
<i>Basiliximab</i> : A chimeric mouse-human monoclonal antibody to the α chain (CD 25) of the IL-2 receptor of T cells.
<i>Daclizumab</i> : Therapeutic humanized monoclonal antibody to the alpha subunit of the IL-2 receptor of T cells (similar to Basiliximab).
<i>Note</i> : Table modified from United Network for Organ Sharing (UNOS).

TABLE 8.2
Immunotoxicity as a Result of Lymphoid Tissue Interaction

Chemicals or pharmaceuticals may cause immunotoxicity as a result of interaction with the following lymphoid tissues and organs:
Primary lymphoid tissues:
• Bone marrow (B cells)
• Thymus (T cells)
Secondary lymphoid tissues:
• Spleen
• Lymphatic system
Tertiary lymphoid tissues:
• Bronchus-associated lymphoid tissue (BALT)
• Nasal-associated lymphoid tissue (NALT)
• Mucosa-associated lymphoid tissue (MALT)
• Skin-associated lymphoid tissue (SALT)

**IMMUNOTOXICITY TESTING: NTP
PROGRAM AND TIER TESTING**

The original National Toxicology Program (NTP) testing approach is outlined in Table 8.3.⁷ This approach encompasses tests identified in the landmark papers on risk assessment and

concordance of immune function tests. The utility of the tier approach was confirmed in a series of publications by Luster et al.^{7–12} in which the results of tier testing with known immunosuppressant agents, as well as a number of industrial and environmental chemicals, were compared with the ability of these compounds to alter host resistance. These studies

provided information on the sensitivity and predictability of the various functional assays.

Concordance Tests: Individual and pairwise concordance tests were used to establish predictability using the immune assays listed in Table 8.4.^{10–12} Concordance was established on the basis that (a) the test material resulted in a dose response or altered two or more immune assays, or (b) compared with host resistance assays since the primary responsibility of the immune system is to protect against infectious or neoplastic disease. High concordance was observed when measuring anti-sheep red blood cell (anti-SRBC) responses and surface marker lymphocyte populations. The results when enumerating surface markers were primarily related to changes in T lymphocyte populations and CD4:CD8 ratios rather than B cell numbers. There were no indications, except for these two tests, that there were other tests individually in the battery that were remarkable predictors for immunotoxicity. In fact, several tests were poor predictors of immunotoxicity.

TABLE 8.3

Original National Toxicology Program Tier Testing Approach

Screen—Tier I	
Immunopathology	Hematology Weights: body, spleen, thymus, Cellularity—spleen Histology—spleen, thymus, LN
Humoral-mediated immunity	Enumeration of IgM antibody PFC Lymphocyte blastogenesis to LPS
Cell-mediated immunity	Mixed lymphocyte reaction (MLR) Lymphocyte blastogenesis to mitogens
Innate nonspecific immunity	Natural killer (NK) cell activity
Comprehensive—Tier II	
Immunopathology	Quantitation of splenic B and T cell numbers
Humoral-mediated immunity	Enumeration of IgG antibody PFC
Cell-mediated immunity	Cytotoxic T lymphocyte (CTL) assay Delayed-type hypersensitivity (DTH) assay
Innate nonspecific immunity	Macrophage function: quantitation of resident peritoneal cells and phagocytic activity
Host resistance challenge models	Viral models, bacterial models, parasite models, and syngeneic tumor cell models (B16F10; PYB6)

Source: Modified from Luster, M.I. et al., *Fund. Appl. Toxicol.*, 10, 2, 1988.

TABLE 8.4

Immune Assays Used to Establish Concordance

Immune assays used to establish individual and pairwise concordance:

Plaque-forming cells (PFC)
NK cell activity
T cell mitogens
Mixed lymphocyte reaction (MLR)
Delayed hypersensitivity reaction (DHR)
Cytotoxic T lymphocyte (CTL)
Surface markers
Leukocyte counts
Thymus/body weight ratio
Spleen/body weight ratio
Spleen cellularity
Lipopolysaccharide (LPS) response

IMMUNOTOXICITY TESTING: A RECOMMENDED APPROACH

Standard toxicity studies (STS; Table 8.5), are surrogates for functional assays which are surrogates for host resistance assays. Many immunotoxicologists believe host resistance assays are best at predicting adverse effects in humans¹³ and the only way to evaluate immunological reserve.^{14–16} In practice, STS often provide the first indication that a chemical or pharmaceutical may be immunotoxic by demonstrating an adverse effect on one or more immunological parameters. In such an event, the STS are followed by functional assays. If a defect is noted in immune function, a host resistance evaluation allows an assessment of the potential of this defect to alter the ability of the host to fight infectious or neoplastic disease.

TABLE 8.5

Standard Toxicity Studies

Standard toxicity studies (STS) may provide the first indication that a chemical or pharmaceutical may be immunotoxic. STS include the following:

Hematology
Clinical chemistry
Gross pathology
Organ weights
Histology

FUNCTIONAL ASSAYS

Functional assays allow further assessment of the defect(s) in immunological cells and/or organs noted during the STS tests, and help determine whether the noted defect(s) results in altered immunological function. Functional assays should include measurements of each of the three arms of the immune system:

1. Innate immunity (Table 8.6)
2. Cell-mediated immunity (CMI) (Table 8.7^{17,18})
3. Humoral-mediated immunity (HMI) (Table 8.8^{14,15,19,20})

TABLE 8.6

Functional Assays—Innate Immunity

Innate immunity: Early nonspecific immune response that consists of nonspecific immunological mediators that recruit and activate innate immune cells

Cytokines/Chemokines

Cytokines and chemokines are nonspecific immunological mediators that are important in cell–cell communication among cells of the immune system. Interferons (IFNs) are important cytokines with immunological mediator and messenger activity, calling in and activating various cells of the immune system. IFNs have potent antiviral, immunomodulatory, and antitumor effects. The different IFN types are alpha, beta, gamma, lambda, and omega.

Natural killer (NK) cells

NK cells have an important role in the interaction of different immunological cell types and cell functions that are important in immunological defense against viral, bacterial, parasitic, and neoplastic disease. The measurement of NK cell activity is an important functional assay of innate immunity following exposure to both pharmaceutical and environmental test articles. Measurement of an antigen- or infectious microorganism-driven cytokine-enhanced immunological response is very important, and measures not only the static activity, but also the ability to be stimulated by an infectious disease. NK cells connect innate and adaptive, acquired immunity. One example with multiple subparts is the production of IFN γ by NK cells, whereby IFN γ acts on NK cells to result in additional production of IFN γ , acts on macrophages to enhance MHC expression, enhance antimicrobial activity, increase antitumor activity, increase TNF α synthesis, increase Fc γ R expression and migration inhibition. IFN γ acts on B cells to alter IgG subclass production, decrease CD23 expression, decrease proliferation, decrease IgE production, and counteract the effect of IL-4. IFN γ acts on T cells to induce IL-2 and IL-2R, alter DTH and graft rejection, enhance suppressor cell activity, and enhance cytotoxicity. IFN γ decreases antiviral replication due to its antiviral effect.

Macrophage, neutrophils, and dendritic cells

Macrophage activity: Macrophages are present and can be measured in many compartments of the body. By virtue of their location throughout the body and their specialized functions, these cells facilitate a critical early interaction with environmental chemicals or pharmaceutical agents. Macrophages are important contributors to early nonspecific innate immunity and also participate in specific immunological responses. Macrophages can initiate and modulate both specific and nonspecific immunological responses. This is due to the following functions of macrophages: processing and presentation of antigen, phagocytosis of infectious agents, killing of infectious agents via respiratory burst, cytostasis and cytotoxicity of tumor cells, production and secretion of a variety of soluble mediators, cytokines including interferon and tumor necrosis factor, chemokines, eicosanoids, chemotactic factor for neutrophils, superoxide anion, and numerous enzymes.

TABLE 8.7

Functional Assays—Cell-Mediated Immunity (CMI)

CMI: Adaptive immune response in which antigen-specific cytotoxic T lymphocytes mediate the cell killing of intracellular pathogens or tumor cells.

Cytotoxic T lymphocyte (CTL) activity: The CTL response is a component of the specific or acquired immune response and has been used to evaluate CMI following exposure to pharmaceuticals or environmental chemicals.¹⁷ The CTL response requires the interaction of the following categories of immune cells:

1. Professional antigen-presenting cells such as dendritic cells and/or macrophages
2. CD4⁺ T-lymphocytes that produce help for response to T-dependent antigens
3. CD8⁺ T-lymphocytes that develop into antigen-specific cytotoxic effector cells.

Antigen presentation is by both class I and class II molecules of the major histocompatibility complex (MHC) to generate effector cytotoxic CTLs. For this reason, the CTL response is distinguished from the delayed-type hypersensitivity (DTH) response or the T-dependent antibody response (TDAR) both of which require only class II presentation of antigens. There are additional differences between the DTH and CTL that have been described when protein or nonreplicating antigens are investigated. Nonreplicating antigens can induce either optimum DTH or an optimum antibody response and has been termed immune deviation.¹⁸ This cross-regulation does not allow an optimum DTH and humoral antibody response in the same animal, and does not allow the immunotoxicological analysis of DTH and TDAR in the same animal.

TABLE 8.8

Functional Assays—Humoral-Mediated Immunity (HMI)

HMI: Is characterized by the production of antibodies, including IgM and IgG. The humoral antibody response may be classified as either TDAR or TIAR.

T-dependent antibody response (TDAR): The TDAR response requires and measures the functionality of three major immune cells: T cells, B cells, and the antigen processing and presentation abilities of dendritic cells and macrophages. Measurement of TDAR is important in assessing the ability of the host to produce antibody. The antibody produced is important in preventing infection by infectious microorganisms, if previously immunized, or can be an important immunological therapeutic in limiting spread and eliminating infectious agents. Class switch can also be evaluated if analysis includes both the IgM and the IgG antibody response. TDAR may be measured by evaluating the number of antibody-forming cells (AFCs) in the spleen following immunization with sheep red blood cells (SRBCs). The SRBC AFC assay uses a technique of Jerne and Nordin.¹⁹ TDAR may also be measured by immunizing with keyhole limpet hemocyanin (KLH) or SRBC and measuring anti-KLH or anti-SRBC in the serum by ELISA. The ELISA method measures antibody in the serum from immunized animals and therefore results in a more thorough evaluation of antibody production in the spleen, lymph nodes, and bone marrow, rather than only in the spleen for the AFC method.²⁰

T-independent antibody response (TIAR): The TIAR response is an important antibody response to polysaccharide antigens such as those on the encapsulated bacteria that cause bacterial pneumonia. This antibody response occurs in the absence of T cell help and requires the presence of marginal zone B cells.^{14,15}

HOST RESISTANCE ASSAYS

If the results of the functional assays that extended the finding(s) from the STS tests indicate that immunological function was altered, host resistance assays can provide an evaluation of the functional defect in the context of the ability of the host to clear an infection or prevent/reduce neoplasms. Namely, is the functional defect adverse, or is there sufficient redundancy/immunological reserve to allow the host to respond normally to the infectious/neoplastic challenge.

The influenza host resistance model is the most thoroughly described infectious disease model and can be performed in either mice or rats in a 28-day repeat dose study.^{14,21,22} Virus is used to infect mice, and viral clearance is measured at designated time points over the 28-day study. Clearance of the infectious agent is the cumulative effect of the orchestrated immune response and is the best method for evaluating the overall health of the immune system. A number of mechanistic immunological components can be evaluated and are shown in Table 8.9.^{16,21,22}

Additional host resistance assays are not required unless there is a specific reason or cause for concern. Targeted host resistance assays, such as the marginal zone B (MZB)-cell assay are performed to answer specific questions regarding the immune response. Targeted host resistance assays that may be evaluated to answer specific questions are listed in Table 8.10.^{14,21–24}

TABLE 8.9

Influenza Virus Host Resistance Assay

Host Resistance Assay to Evaluate the Overall Health of the Immune System: Influenza Virus in Mice or Rats^a

The following are mechanistic immunological function endpoints that can be evaluated in this model:

- Cytokines—innate immunity
- Interferon activity—innate immunity
- Macrophage activity—innate immunity
- NK cell activity—innate immunity
- CTL activity—cell-mediated immunity (CMI)
- Influenza-specific IgM, IgG (IgG1 and IgG2a)—TDAR—humoral-mediated immunity (HMI) (TDAR)
- Immunophenotyping
- Histopathology

^a Burleson and Burleson.^{16,21,22}

TABLE 8.10

Targeted Host Resistance Assays

Marginal Zone B (MZB) Cell Assay^{14,21,22}

Indication: Concern arising from histopathology results indicating an effect of the test article on the spleen marginal zone. Histopathology and extended histopathology studies are crucial to the detection of immunotoxicity.^{23,24}

- MZB cells are required for T-independent antibody response (TIAR).
- TIAR is the immune response to polysaccharide antigens such as the capsular antigens of encapsulated bacteria that are responsible for numerous diseases including bacterial pneumonia.
- Systemic *Streptococcus pneumoniae* host resistance assay measures
 - Antibody to *S. pneumoniae*
 - Bacterial clearance

S. pneumoniae Pulmonary Assay for Innate Immunity^{14,21,22}

Indication: Concern arising from a defect in innate immunity parameters, especially macrophages and neutrophils. This assay measures bacterial clearance at 24 h, a time when acquired immune functions have not yet developed.

This host resistance assay can be used to evaluate:

- Therapeutics affecting neutrophils and/or macrophages
- Anti-inflammatory therapeutics
- Therapeutics targeting TNF α

Listeria Monocytogenes Systemic Assay^{14,21,22}

Indication: Concern arising from a defect in cell-mediated immunity. This assay measures bacterial clearance at multiple time points over 7 days.

- Intracellular Gram-positive bacterial assay to evaluate liver and splenic macrophages and neutrophils
- Evaluates cell-mediated immunity (CMI)

Murine Cytomegalovirus (MCMV) Latent Viral Reactivation Assay^{14,21,22}

Indication: Concern arising from a decrease in CMI that could result in reactivation of latent viral infection.

- Host resistance measures viral reactivation of a latent infection.

Candida Albicans Assay^{14,21,22}

Indication: Concern arising from defects in antifungal immunity.

- Measures clearance of infectious *Candida albicans*

NONHUMAN PRIMATES:

IMMUNOTOXICOLOGICAL EVALUATIONS

Human biologicals are now an important part of the pharmaceutical pipeline with many now approved and used in prevention and/or therapy of human diseases. This drug development armamentarium of biotech and pharmaceutical companies must be thoroughly evaluated for immunotoxicity.

Many human biologicals are fusions of IgG, and/or target immune mediators, immunological receptors, adhesion molecules, and/or are indicated for diseases that have immune components. It is therefore necessary to thoroughly evaluate human biological therapeutics for immunotoxicity. Numerous biologicals that are pharmacologically active in rodents can be evaluated using well-characterized rodent host resistance assays. However, biologicals not active in rodents may use surrogate biologicals for testing in rodent host resistance assays, or may use host resistance assays in genetically engineered mice that mimic the effect of the human biological pharmacological agent. There are approaches for immunotoxicological evaluations being developed and refined for nonhuman primates. It is important that immunotoxicological evaluations in nonhuman primates be no less rigorous than in rodents, and include evaluations of the three arms of the immune response.

IMMUNOTOXICITY TESTING: REGULATORY

Regulatory expectations for immunotoxicity testing are provided by the US EPA (Table 8.11) for chemicals as guidelines and by the US FDA (Table 8.12) as guidances.

TABLE 8.11

Regulatory: Chemicals

Pesticide Immunotoxicity Testing

US EPA OPPTS 870.7800 Immunotoxicity, August 1998²⁵

40 CFR Parts 9 and 158 Federal Register Notice/Vol. 72, No. 207, October 26, 2007²⁶

T-dependent antibody response (TDAR)

28-day repeat dose toxicity test

At least three dose levels

Positive control

Vehicle control

Sheep red blood cell (SRBC) functional assay

ELISA or splenic PFC assay

Natural killer (NK) cell functional assay

Multiple effector: target cell ratios

YAC-1 target cells

Spleen effector cells

⁵¹Cr-release assay

Note: If the test article produces dose-related suppression of the TDAR (anti-SRBC response) without evidence of overtotoxicity to nonimmune system, then the test article is considered as immunotoxic and no additional studies are required. If the TDAR is negative and standard toxicity studies (STS) (e.g., changes in lymphoid organ weights, hematology, histopathology) suggest evidence of immunotoxicity, then NK cell activity may need to be evaluated as a measure of non-specific (innate) immune function. In contrast, if the TDAR assay is negative and evaluation of STS provides no evidence of immunotoxicity, then the test article is considered negative for immunotoxicity and evaluation of NK activity is not necessary. Finally, if the TDAR is negative and limited or no observational immune system end points are available, an NK assay may need to be performed.

TABLE 8.12

Regulatory: Pharmaceuticals

ICH S8 Immunotoxicity Testing Guidance for Industry

Immunotoxicity studies for human pharmaceuticals

ICH April 2006²⁷

The following is helpful in addressing immunotoxicological concerns:

Standard toxicity studies

- Hematology
- Clinical chemistry
- Gross pathology
- Organ weights
- Histology

Selection and design of immunotoxicity studies:

1. Objectives
2. Selection of assays
3. Study design
4. Evaluation of additional immunotoxicity studies and recommendations concerning further studies

Timing of immunotoxicity testing in relation to clinical studies:

1. If weight-of-evidence review indicates that additional immunotoxicity studies are appropriate, these should be completed before exposure of a large population of patients—usually Phase 3.
2. The timing might be determined by the nature of the effect and the type of clinical testing that would be required if a positive finding is observed.
3. Immunotoxicity testing can be initiated at an earlier time point in the development of the drug if the target population is immunocompromised.

Guidance for Industry

ICH S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals

ICH July 1997²⁸

and

Guidance for Industry

ICH S6 Addendum to Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals

ICH May 2012²⁹

The following is helpful in addressing immunotoxicological concerns for large molecule biologics:

Section IV. Immunogenicity assessments are conducted to assist in the interpretation of the study results and design of subsequent studies.

IMMUNOENHANCEMENT

Immunoenhancement may either be intended or unintended. Certain therapeutics are designed to cause immune system activation (Table 8.13). Other chemicals or pharmaceuticals may cause a direct unintended immunostimulation or hypersensitivity reaction (Table 8.14). Even pharmaceuticals designed to enhance the immune response may cause unintended immunotoxicity, as was the case with the cytokine storm syndrome caused by TGN-1412.^{30–32} Unintended immunostimulation can also result in hypersensitivity reactions and autoimmune disease.

TABLE 8.13
Examples of Intended Therapeutic Immunostimulation

Biological Therapeutics Associated with Immune System Activation

IL-2
IL-6
IL-12
IL-18
Anti-CD52 mAb
Alemtuzumab (Campath®)
Anti-CTLA-4
Ticilimumab and ipilimumab
Rhu-IFN- α_{2a} and Rhu-IFN- α_{2b} (PEGASYS®, ROFERON®-a, iNTRON®-a, peg-iNTRON®)
RHU-gm-csf
Sargramostim (Leukin®)
Anti-CD3 mAb
Moromonab-CD3 (Orthoclone OKT®3)
Anti-CD20 mAb (Rituximab [Rituxan®])
TGN-1412

Source: Modified from Gribble, E.J. et al., *Expert Opin. Drug Metab. Toxicol.*, 3, 209, 2007.

HYPERSENSITIVITY

Hypersensitivity reactions occur when the immune system directs its efforts and effects on self-molecules, proteins, cells, and tissues. These misguided immune responses can manifest as a result of bystander reactions to native

proteins, molecular mimicry on the part of chemicals/drugs and pathogens, and haptenation of native proteins by chemicals. Hypersensitivity develops in several phases. Initial exposure to the offending agent establishes recognition by the immune system. Overt toxicity and tissue damage may exacerbate the immune response to initial exposure, resulting in an adjuvant effect that supports maturation of an adaptive immune response to native or altered native proteins. The next phase involves re-exposure to the offending agent, which results in the recruitment of lymphocytes and other immune cells important for establishing an allergic response. Finally, if exposure persists, the allergic inflammation at the site of exposure will lead to the symptoms of hypersensitivity. In the skin, these symptoms often include development of a rash, blistering, urticaria, itching, and burning sensations. When exposure is through inhalation, symptoms manifest in the airways as difficulty breathing, wheezing, coughing, chest tightness, and congestion in the lower airways. In the upper airways, hypersensitivity responses lead to rhinorrhea, itchy nose, congestion, sneezing, and itchy and watery eyes. The underlying mechanisms involve recruitment of immune cells to the site of exposure and release of mediators that can cause tissue damage and remodeling. Table 8.14 provides a list of the types of hypersensitivity reactions that can occur and some of the agents known to induce each type. The remainder of this section will provide an overview of the mechanisms, agents, and hazard identification testing strategies for dermal and respiratory hypersensitivities.

TABLE 8.14
Examples of Unintended Immunostimulation/Hypersensitivity

Examples of the Four Types of Hypersensitivity Responses ^a				
Agents: Clinical Manifestations	Hypersensitive Reaction	Cells Involved	Antibody	Mechanism of Cell Injury
Food additives: GI allergy Penicillin: urticaria and dermatitis Anhydrides: occupational asthma	Type I (anaphylactic)	Mast cell	IgE (and others)	Degranulation and release of inflammatory mediators such as histamine, proteolytic enzymes, chemotactic factors, prostaglandins, and leukotrienes
Cephalosporins: hemolytic anemia Aminopyrine: leukopenia Quinidine, gold: thrombocytopenia	Type II (cytotoxic)	Null (K) cells ^b	IgG, IgM	Antibody-dependent cellular cytotoxicity, or complement-mediated lysis
Hydralazine: systemic lupus erythromatosis Methicillin: chronic glomerulonephritis	Type III (immune complex)	PMNs ^c	IgG, IgM	Immune complex deposition in various tissues activates complement, which attracts PMNs causing local damage by release of inflammatory cytokines
Nickel, penicillin, dinitrochlorobenzene, phenothiasines: contact dermatitis	Type IV (delayed hypersensitivity)	T cells (sensitized); macrophages	None	Release of lymphokines activates and attracts macrophages, which release mediators that induce inflammatory reactions

Source: Norbury, K. and Thomas, P.T., *In Vivo Toxicity Testing: Principles, Procedures, and Practices*, Academic Press, New York, 1990. With permission.

^a Hypersensitivity response classification as defined by Coombs, R.R.Z. and Gell, P.G.H.³⁴

^b Also, T cells, monocyte/macrophages, platelets, neutrophils, and eosinophils.

^c Polymorphonuclear leukocytes.

DERMAL HYPERSENSITIVITY

The skin serves as an important barrier between body tissues/fluids and the external environment. Hypersensitivity reactions involving the skin can compromise this barrier, increasing host susceptibility to infectious agents and toxic compounds. Such reactions are usually directed toward low molecular weight (LMW) compounds that are capable of penetrating the complex stratification of the skin and result in delayed (24–48 h) cell-mediated responses. However, immediate hypersensitivity reactions in the skin, commonly referred to as immunologic urticaria, can also occur following sensitization and subsequent skin exposure to high molecular weight (HMW) proteins capable of eliciting specific IgE production. Clinical symptoms observed following secondary exposure to the skin allergens include pruritus and stinging sensations that are accompanied by vesicular and bullous lesions and/or pigment alterations at the site of allergen contact. Inflammatory pustules and secondary bacterial infections can accompany these rashes, which can be progressive and destructive if allergen exposure is not prevented.

Identification of agents with the potential to induce allergic contact dermatitis (ACD) is critical to improving human health risk assessment. Due to national and international regulatory requirements, testing for ACD is common. Originally, the guinea pig was the animal of choice for predictive studies of skin sensitization potential. This arose largely as a result of the use of the guinea pig in the pioneering investigations into mechanisms of skin sensitization to chemicals^{36,37} although the first definition of a real predictive test came from the work of Draize et al.,³⁸ almost 70 years ago. Because of significant animal welfare benefits, the local lymph node assay (LLNA) has been increasingly accepted as the method of choice for identifying potential skin sensitizers. In this method, skin-sensitizing potential is measured as a function of lymph node (LN) cell proliferation induced in mice following repeated topical exposure of the test chemical.³⁹ The OECD test guideline 429⁴⁰ for the LLNA suggests a minimum of three test concentrations and a vehicle control group with a minimum of four animals per group. There are also guidelines published by ICCVAM following extensive validation of the LLNA.⁴¹ More recently, several scientific review committees, including ICCVAM^{42,43} and ECVAM,⁴⁴ following review of extensive validation studies, have recommended the use of a “reduced” LLNA (rLLNA) as it would further decrease the number of animals required for a test

for certain data requirements. The rLLNA requires only a negative control group and the equivalent of the high-dose group from the “full” LLNA. However, the ability to determine dose response and better establish relative potency is limited in the rLLNA, and for most risk assessment purposes the traditional LLNA is preferred. The LLNA determines the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment. Recent recommendations from ICCVAM in 2010 reported that the types of materials that can be assessed accurately using the LLNA include several additional classes of chemicals such as metals, pesticides, and aqueous substances.⁴⁵

The general approach used for conducting hypersensitivity assessment is shown in Figures 8.1 and 8.2. Initially, appropriate dose levels and vehicle are identified with aid from the published literature, government toxicity resources (i.e., NTP), and when necessary, irritancy tests. Dose selection is critical as irritant contact dermatitis (ICD), which can occur at higher concentrations for chemicals that produce hypersensitivity, can interfere with interpretation of the LLNA by causing nonspecific proliferation in the target LNs. Thus, it may be necessary to incorporate an irritancy test as part of the LLNA assay in order to determine the maximal nonirritating concentration to be used as an upper concentration in the LLNA. The irritancy test and LLNA can be conducted in tandem in order to reduce the number of animals that would be required if conducted independently. In fact, OECD 429⁴⁰ now recommends assessment of ear swelling as a routine component of the LLNA to rule out excessive irritation in interpreting the results.

The mouse ear swelling test (MEST), originally described by Gad et al.,⁴⁶ requires considerable utilization of mice as well as injection of Freund's complete adjuvant and is seldom currently used. A modified test has been described by Auttochoat et al.⁴⁷ that is more applicable for testing the dermal sensitizing potential of certain classes of chemicals when it is appropriate to examine the elicitation phase of allergic hypersensitivity. This test involves sensitization of the animal via dermal exposure to the test article on the flank. The sensitized animal is then challenged with the test agent on the dorsum of the ear, and changes in ear thickness are measured as the biological assessment of the elicitation phase of the hypersensitivity response. If the test article is a dermal sensitizer, challenge on the ears will result in swelling due to allergic inflammation.

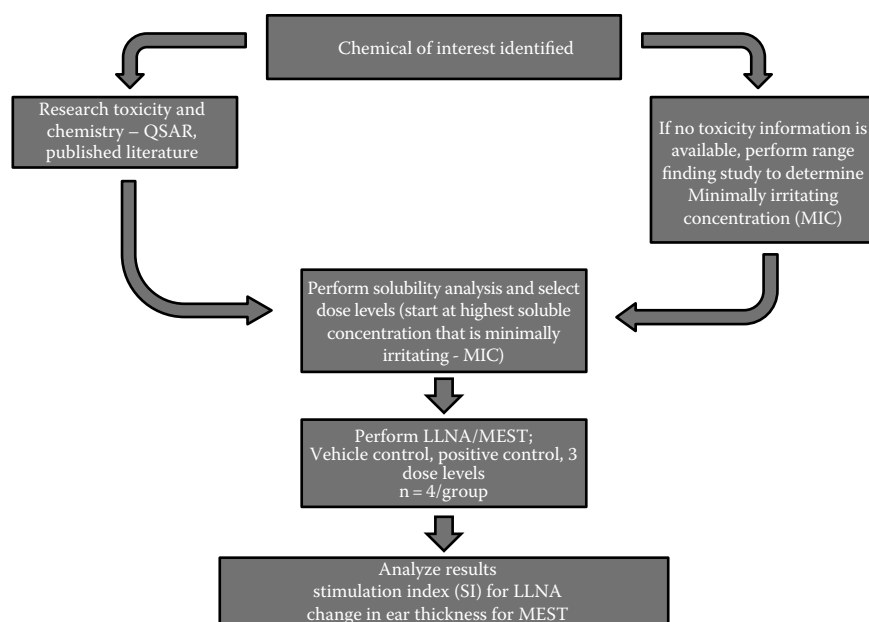


FIGURE 8.1 General approach to dermal hypersensitivity testing. Critical information regarding dose selection should be gathered from all available sources prior to initiation of dermal hypersensitivity testing. It is important to establish the maximal nonirritating concentration, as it is known that excessive irritation can interfere with interpretation of the results due to nonspecific proliferation of lymph node (LN) cells. Immediate ear swelling (within 24 h) can be used as an indication of irritation as well as to distinguish between chemical-induced allergic-contact dermatitis and irritant-contact dermatitis. This assessment can proceed and also be incorporated into both the local lymph node assay (LLNA) (Figure 8.2) and mouse ear swelling test (MEST).

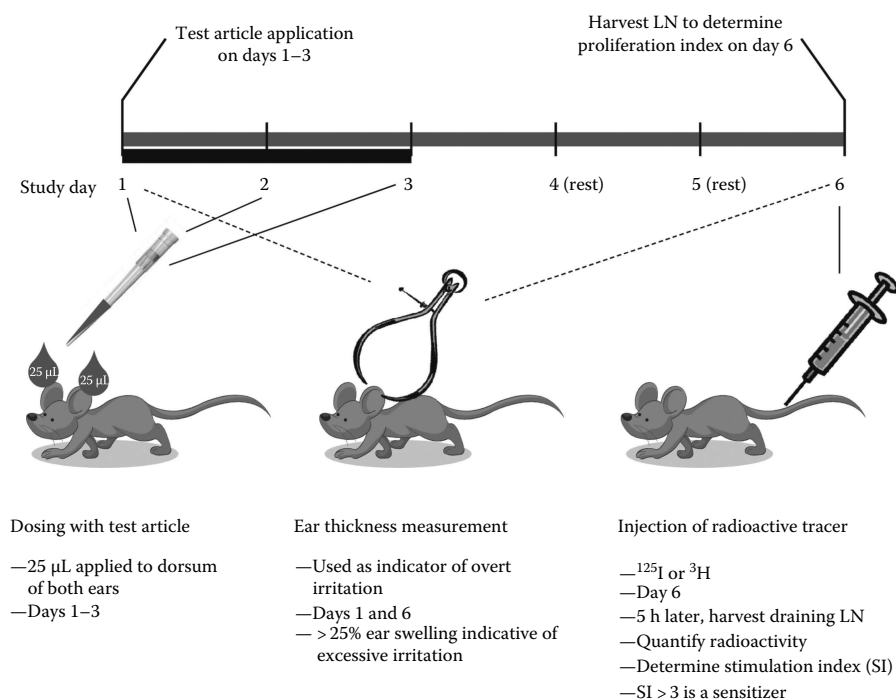


FIGURE 8.2 Local lymph node assay (LLNA). The LLNA is performed according to the guidelines recommended by ICCVAM⁴¹ and OECD 429.⁴⁰ The LLNA evaluates the allergenic potential of a test substance following topical application to mouse ears. Sensitizers cause an increased induction of lymphocyte proliferation in the draining auricular lymph nodes, compared with vehicle controls. The proliferative response is measured by quantifying the incorporation of a tracer into DNA by the proliferating lymphocytes of the auricular LNs. A chemical is classified as a skin sensitizer if at one or more test concentrations induces a threefold or greater increase in draining LN cell proliferation compared with concurrent vehicle-treated controls (Stimulation Index [SI] ≥ 3). In the original LLNA, four mice per treatment group were used. Nonspecific proliferation due to irritation has been known to confound LLNA interpretation. Ear thickness increases of $\geq 25\%$ have been associated with excessive local skin irritation,⁴⁰ and excessive ear irritation has the potential to interfere with the LLNA assessment. Ear thickness measurements will be taken for both ears prior to dosing on Day 1 and also on Day 6. (Images obtained from Microsoft Office Clip Art.)

RESPIRATORY SENSITIZATION: EXPOSURE, EFFECTS, AND AGENTS

The respiratory system serves the vital function of supplying oxygen and removing waste carbon dioxide from tissues with the upper airways filtering and conditioning the air for the lungs where gas exchange occurs. Respiratory hypersensitivity can affect many aspects of airway function ranging from a bothersome runny/itchy nose to disconcerting wheeze and shortness of breath and even fatal airway constriction. Allergen exposure can manifest as an early-phase response, a late-phase response, or a dual response, which are the results of isolated or combined Type I and Type IV hypersensitivity reactions. The early-phase response is primarily driven by allergen-specific IgE antibody originating during sensitization that is bound to surface receptors of mast cells and basophils residing in circulation and respiratory tissue. Inhalation of allergen results in cross-linking of IgE and activation/degranulation of mast cells, leading to the release of preformed mediators such as histamine and leukotrienes. These molecules, among others, elicit the effector responses

of rhinitis in the upper airways (Figure 8.3; sneezing, itching, rhinorrhea, and severe nasal obstruction) or asthma in the lower airways (Figure 8.4; dyspnea, wheeze, coughing, and chest tightness). Development and utilization of testing strategies that provide information analogous to the LLNA have been initiated. Arts et al.⁴⁸ developed a respiratory version of the LLNA in which chemical exposure occurs through inhalation. Similar exposure timeline and assay readouts (i.e., draining LN proliferation) are used. This assay was recently applied to identify ortho-phthalaldehyde as a respiratory sensitizer.⁴⁹ Despite these efforts and the critical need for identification of agents that can cause respiratory hypersensitivity, validation of testing methods is lagging behind that of dermal hypersensitivity. Potential consequences of exposure to agents capable of inducing or influencing sensitization and allergic hypersensitivity in the airways are shown in Figure 8.5. Information presented in this section is focused on respiratory hypersensitivity from the perspective of symptoms, known causative agents (Tables 8.15 and 8.16), and potential testing approaches for hazard identification and risk assessment.

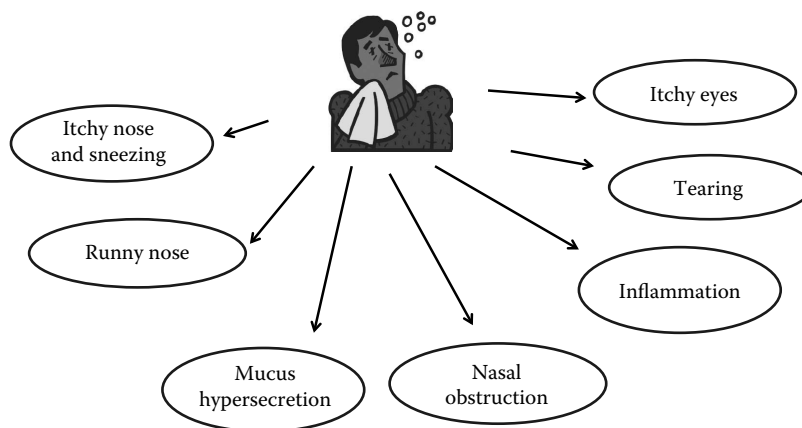


FIGURE 8.3 Allergic rhinitis. Allergic rhinitis sensitization of the upper airways can result in a disease called allergic rhinitis. The symptoms of allergic rhinitis manifest following exposure to the offending agent once sensitization is established. Symptoms include itchy and runny nose, sneezing, nasal obstruction, itchy eyes, and tearing. These symptoms are the result of inflammation, cytokine/chemokine release, immune cell infiltration, histamine/leukotriene release, mucus hypersecretion, and edema. In severe cases, remodeling of the nasal epithelium can occur resulting in fibrosis, decreased sensitivity to smell, chronic obstruction, and perforations. Many agents have been identified as occupational and environmental inducers of allergic rhinitis, and the most commonly reported agents are listed in Table 8.15. (Image obtained from Microsoft Office 2007 Clip Art.)

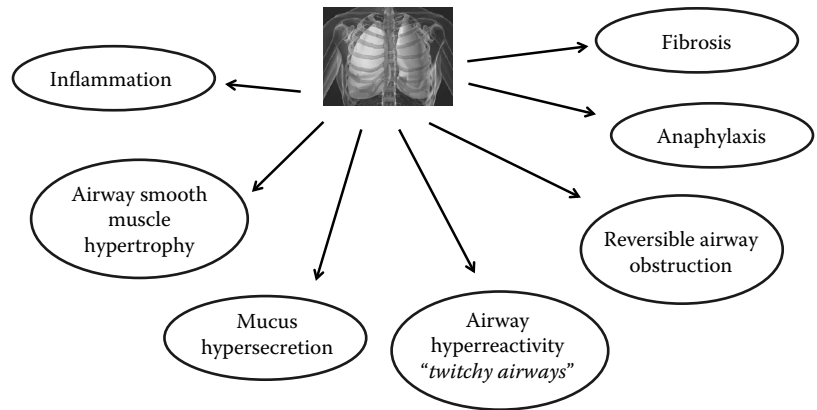


FIGURE 8.4 Allergic asthma. Sensitization of the lower airways can result in a disease called allergic asthma. The symptoms of allergic asthma manifest following exposure to the offending agent once sensitization is established. Symptoms include airway hyper-reactivity, better known as “twitchy airways,” airway obstruction/constriction leading to difficulty breathing, wheezing, and mucus plugging. These symptoms are the result of inflammation, cytokine/chemokine release, immune cell infiltration, histamine/leukotriene release, mucus hypersecretion, smooth muscle hyperplasia, and edema. Antibodies specific for the chemical allergen/hapten also play an important role in disease pathogenesis. Chemical-specific IgE is involved in the immediate hypersensitivity response (Type I reaction), whereas IgG is involved more in the late phase response to exposure and contributes with cellular inflammation to Type IV hypersensitivity in the lungs. In severe cases, remodeling of the respiratory epithelium can occur resulting in fibrosis, increased smooth muscle mass and contractility, chronic obstruction/constriction, and gas trapping. Many agents have been identified as occupational and environmental inducers of allergic asthma, and the most commonly reported agents are listed in Table 8.16. (Image obtained from Microsoft Office 2007 Clip Art.)

TABLE 8.15
Agents Known to Cause Occupational Rhinitis

Agents	Occupation/Exposure
<i>High molecular weight agents</i>	
Laboratory animals	Laboratory workers/pet rodents
Other animal-derived allergens	Swine confinement workers
Insects and mites	Laboratory workers, farmers, household dusts
Grain dust	Grain elevator workers, farmers, farm families
Flour	Bakers
Latex	Hospital workers, textile factory workers
Other plant allergens	Grasses, trees, tobacco, hot pepper, tea, coffee, cocoa, dried fruit, saffron
Biological enzymes	Pharmaceutical and detergent manufacturing, household detergent use
Fish and seafood proteins	Many fish and shellfish, fishermen, fish/shellfish farmers, processing workers
<i>Low molecular weight agents</i>	
Diisocyanates	Painters, urethane mold workers, foam production, insulation, hobby word workers
Anhydrides	Epoxy resin production, chemical workers, electric condenser workers
Wood dust	Carpenters and wood workers, furniture manufacturing, woodworking hobbyist
Metals	Platinum refinery
Drugs (psyllium, spiramycin, piperacillin)	Healthcare and pharmaceutical workers
Chemicals	Reactive dyes, synthetic fiber, cotton, persulfate, hairdressing, pulp/paper, shoe manufacturing, metal working fluid

Source: Adapted from Moscato, G. et al., *Resp. Res.*, 10, 16, 2009.

TABLE 8.16
Chemicals Known to Cause Respiratory Hypersensitivity Resulting in Allergic Asthma in Humans

Chemical Name	Alternate Name	Occupation or Industry
<i>Acrylates and methacrylates</i>		
Ethyl cyanoacrylate	Ethyl-2-cyanoacrylate	Building airplane models
Methyl 2-cyanoacrylate		Using adhesives
Ethyl methacrylate		Manicurist
Methyl methacrylate		Nurse
Ethoxylated bisphenol A diacrylate		Autobody shop worker
<i>Metals</i>		
Aluminum		Solderer
Chromium and compounds		Printer, plater, welder, tanner
Cobalt		Hard metal grinder, diamond polisher
Nickel and compounds		Metal plating; welder
Palladium		Assembly line worker
Platinum		Platinum refinery
Tungsten carbide		Grinder
Zinc chloride fume		Solderer
<i>Aldehydes</i>		
Formaldehyde		Hospital staff
Glutaraldehyde	Cidex	Hospital endoscopy unit
Ortho-phthalaldehyde	Cidex OPA	Hospital endoscopy unit
<i>Amines</i>		
Ethylenediamine	1,2-diaminoethane	Shellac handler; photographer
Hexamethylenetetramine		Lacquer handler
<i>N,N</i> -Dimethyl-1,3-propanediamine	DMAPA	Ski manufacturer
Triethylenetetramine		Manufacturing aircraft filters
EPO 60		Mold maker
Trimethylhexanediamine + Isophorondiamine		Floor covering material salesman
4-Methylmorpholine		Manufacturing polyurethane foam
Piperazinedihydrochloride		Pharmaceutical and chemical manufacturing
<i>p</i> -Phenylene diamine		Fur dyeing
Ethanolamine	2-Aminoethanol	Beauty culture
<i>N,N</i> -Dimethylethanolamine		Spray painter
<i>N</i> -(2-hydroxyethyl)ethylenediamine	Aminoethylethanolamine	Solderer; cable jointer
Triethanolamine		Metal worker
<i>Acid anhydrides</i>		
Chlorendic anhydride		Mechanic
Hexahydrophthalic anhydride		Chemical worker
Himic anhydride		Manufacturing flame retardant
Maleic anhydride		Manufacturing polyester resin
Methyltetrahydrophthalic anhydride		Using curing agent
Phthalic anhydride		Producing resins
Pyromellitic dianhydride		Using epoxy adhesives
Tetrachlorophthalic anhydride		Using epoxy resins
Trimellitic anhydride		Using epoxy resins
<i>Preservatives/disinfectants</i>		
Benzalkonium chloride		Using cleaning product
1,2-Benzisothiazolin-3-one		Chemical manufacturing
Chloramine T		Chemical manufacturing; brewery; janitorial/cleaning
Chlorhexidine	Hibiclens	Nurse
Hexachlorophene		Hospital staff

TABLE 8.16 (continued)

Chemicals Known to Cause Respiratory Hypersensitivity Resulting in Allergic Asthma in Humans

Chemical Name	Alternate Name	Occupation or Industry
Isononanoyl oxybenzene sulfonate		Laboratory technician
Lauryl dimethyl benzyl ammonium chloride		Using floor cleaner
Methylchloro-isothiazolinone		Chemical manufacturing
<i>Isocyanates</i>		
Dicyclohexylmethane 4,4-diisocyanate	Hydrogenated MDI	Manufacturing polyurethane products
Hexamethylene diisocyanate	HDI	Spray painter
Isophorone diisocyanate	IPDI	Spray painter
Methylene bisphenyl isocyanate	MDI; diphenylmethane diisocyanate	Foundry
Naphthalene diisocyanate	NDI	Rubber manufacturing
Polymethylene polyphenyl isocyanate	PPI	Paint shop worker
Toluene diisocyanate	TDI	Producing polyurethanes; floor varnisher
<i>Plastic and rubber dusts</i>		
Azodicarbonamide	1,1'-Azobisformamide	Rubber and plastic manufacturing
Plexiglass (dust)	Lucite; methyl methacrylate polymer;	Factory worker
Polyvinyl chloride (heated)		Meat wrapper's asthma
Polyvinyl chloride (dust)		Manufacturing bottle caps
Polyethylene (heated)		Paper wrapper's asthma
Polypropylene (heated)		Manufacturing bags
<i>Pyrolysis products</i>		
Rosin core solder	Rosin flux pyrolysis products	Electronics worker; manufacturing solder flux
Zinc chloride fume		Solderer; locksmith
<i>Fungicides</i>		
Bis(tri- <i>n</i> -butyltin)oxide	Tributyltin oxide	Exposure to carpet deodorizer
Captafol	Difolatan	Chemical manufacturing
Chlorothalonil	Tetrachloroisophthalonitrile	Farmer
<i>Other chemicals</i>		
Aluminum smelting	Yet to be identified substance or mixture (aluminum, fluorides) that can cause "potroom asthma" in workers at electrolytic reduction facilities	Potroom worker
Ammonium persulfate		Hairdresser
Diazonium salt	Example, diazonium tetrafluoroborate and p-diethylaminobenzenediazonium chloride	Manufacturing photocopy paper; manufacturing fluorine polymer precursor
Dioctyl-phthalate		PVC production worker
Drugs		Pharmacist; pharmaceutical worker
Ethylene oxide		Nurse
Furfuryl alcohol		Foundry mold making; wool dye house
Ninhydrin		Laboratory worker
Nitrogen chloride		Indoor pool lifeguards
Oil mist, mineral	Metalworking or machining fluids, cutting oils (may contain numerous additives and contaminants)	Toolsetter and automobile plant
Styrene		Plastics factory
Sulfites		Water plant; food processor
Tetramethrin		Exterminator
Tetrazene		Detonator manufacturing
Textile dyes	Reactive dyes, Levafix brilliant yellow E36, Drimaren brilliant yellow K-3GL, Cibachrome brilliant scarlet 32, Drimaren brilliant blue K-BL, Lanosol yellow 4G, Carmine, Black henna, Monascus ruber (food colorant), FD&C blue dye #2	Textiles, dye manufacturing
Triglycidyl isocyanurate		Spray painter
Urea formaldehyde	Kaurit S	Resin and foam manufacturing

Source: Adapted from Chan-Yeung, M. and Malo, J.L., *Eur. Resp. J.*, 7, 346, 1994.

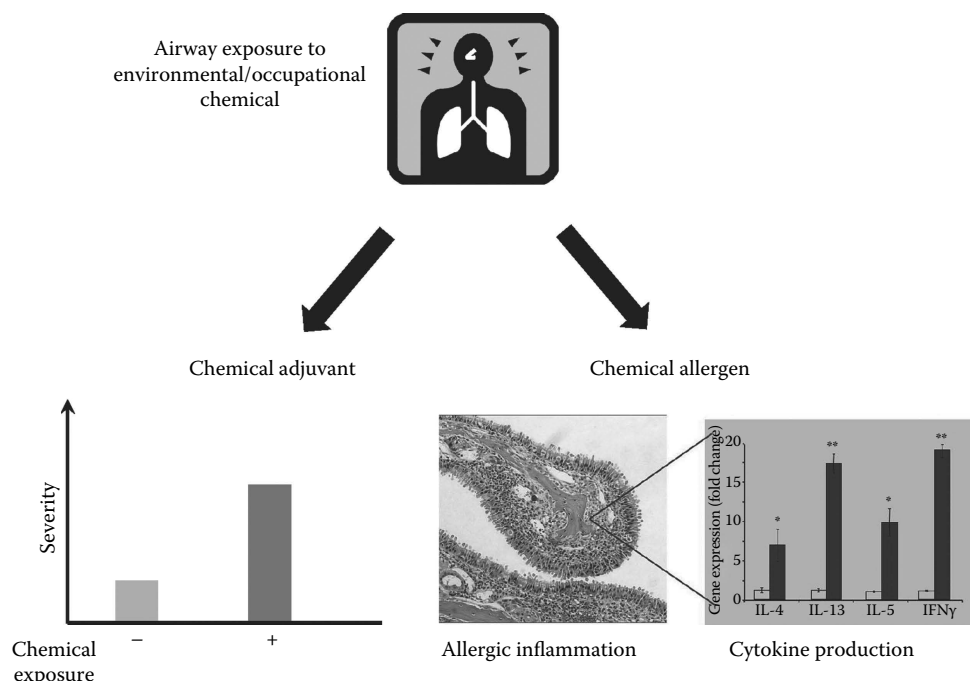


FIGURE 8.5 Airway exposure to chemicals. Exposure to chemicals in the workplace and environment can affect the airways in two ways. First, the chemicals can act as adjuvants, where exposure to the chemical can facilitate sensitization to other agents and exacerbate hypersensitivity reactions to known sensitizers. Such chemicals can lead to airway sensitization to agents that would not normally cause respiratory hypersensitivity at exposure levels encountered in the environment or workplace. Second, chemicals can be complete sensitizers where exposure leads to sensitization to the chemical. Subsequent exposures to the chemical result in the elicitation of symptoms of allergic rhinitis (see Figure 8.3) and/or allergic asthma (see Figure 8.4). (Image obtained from Microsoft Office 2007 Clip Art.)

AUTOIMMUNITY

Autoimmunity is a disease condition in which normal self-tolerance is broken, resulting in immune responses against self-antigens. These misguided immune responses contribute to the systemic and organ-specific pathologies that characterize autoimmune diseases. Development of autoimmune disease is normally a slow evolving process and it is likely to require an extended period for histopathological or serum biomarker changes to be expressed. In addition, susceptibility to most autoimmune diseases is dependent on polygenic inheritance, environmental factors, and poorly defined stochastic events. One of the significant challenges facing autoimmune disease research is in identifying the specific events that trigger loss of tolerance and autoimmunity, and for immunotoxicology, developing screening tests that can accurately predict the potential for agents to initiate or influence preexisting autoimmune disease. Traditional approaches to identify such agents include undertaking exposures in autoimmune prone rodents and monitoring for the accelerated and/or exacerbated development of autoimmune disease as indicated by elevated levels of serum autoantibodies and/or Ig levels as well as histopathological changes in the target organ(s) or changes in serum chemistries. Historically, MLR autoimmune-prone mice and to a lesser extent lupus-prone NZM and NZB \times NZW (F1) mouse strains have been used. Although the accuracy of this approach has yet to be established, much

of its impetus is based upon studies where certain classes of chemicals, such as organic solvents (e.g., trichloroethylene), have been shown to exacerbate development of autoimmune disease in genetically prone rodent models.⁵² Other genetically prone animal models that have been employed, with varying levels of success, include the NOD mouse, a model for autoimmune diabetes; NZB and MLR mice, which are systemic lupus-prone; and Brown Norway (BN) rats, models for mercury-induced glomerulonephritis. There are a number of genetically prone animal test models that can be used depending upon the suspect target organ (Table 8.17). Animal models have been discussed more comprehensively in the World Health Organization (WHO) document "Principles and methods for assessing autoimmunity associated with exposure to chemicals."⁵³

Although a number of syndromes similar to those observed clinically in humans can be mimicked in animal models, the diversity of immune mechanisms and pathogenesis of autoimmune diseases limit the utility of any single model as a screening tool, and validated tests do not exist. There is a pressing need for development of predictive screening assays for identification and prioritization of chemicals with the potential to cause autoimmunity. Such assays might need to be based on multiple outcomes from long-term chemical exposure studies since there are many types of autoimmune disease (over 30), and they can take years to decades to develop in humans. More recent efforts suggest a tiered approach to autoimmunity

TABLE 8.17
Examples of Autoimmune-Prone Rodent Strains Available to Identify Autoimmune Disease Acceleration

Strain	Disease	Target
<i>Spontaneous autoimmune disease models</i>		
B6;129S6-Gadd45b ^{tm1Flv} /J	Experimental autoimmune encephalomyelitis	CNS
BBDR/Wor	Type 1 diabetes	Endocrine, pancreas
MRL/MpJ-Fas ^{lpr} /2J	Arthritis, immune complex glomerulonephrosis, SLE	Kidney, liver
NZBxNZW F1	SLE	Kidney, ANA
B6.Cg-Fbn1 ^{Tsk} /J	Scleroderma	Skin
<i>Induced autoimmune disease models</i>		
SJL	MBP- or spinal cord homogenate-induced autoimmune encephalitis animal model of multiple sclerosis	CNS
DBA/1	Collagen-induced arthritis animal model of rheumatoid arthritis	Joints
BN Rat	Chemical (i.e., mercuric chloride, D-penicillamine, trichloroethylene)-induced SLE like syndrome	Kidney, skin, ANA

Notes: CNS, central nervous system; SLE, systemic lupus erythromatosis; MBP, myelin basic protein; ANA, antinuclear antibodies.

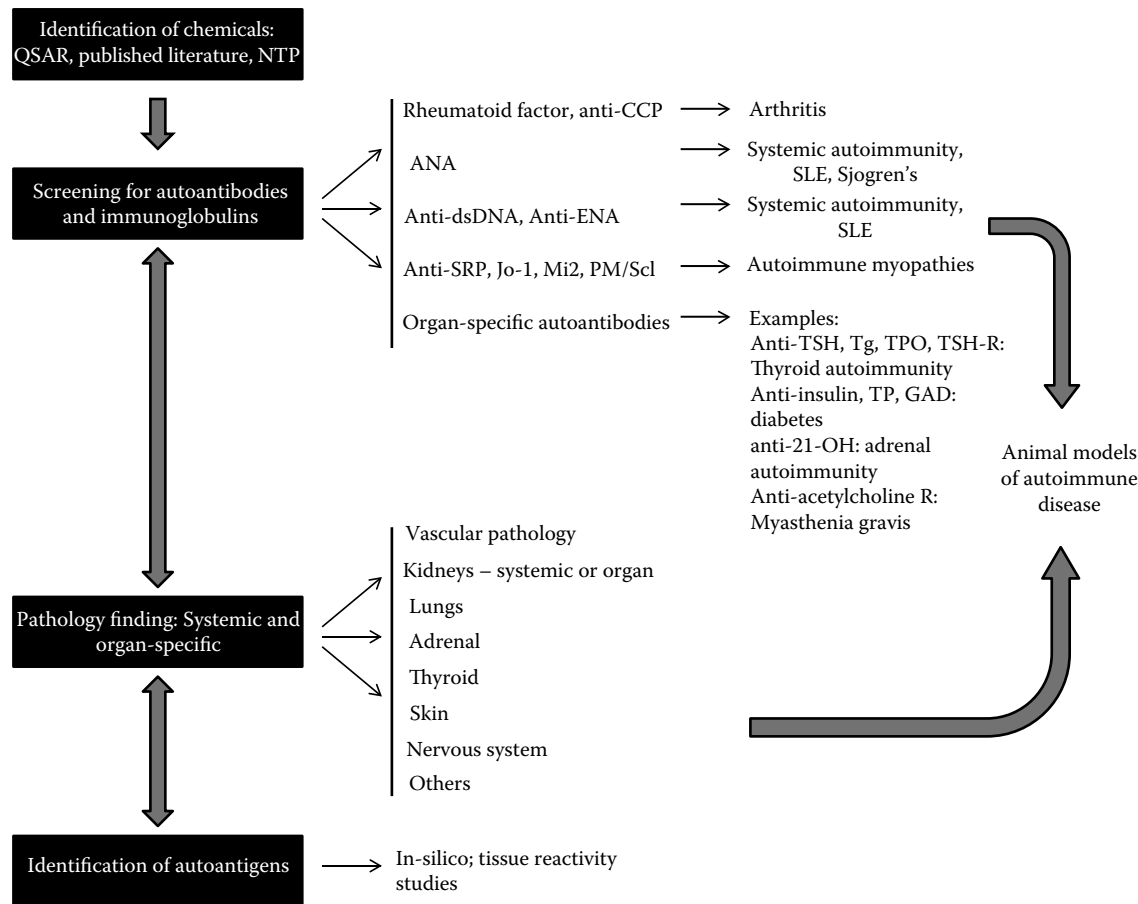


FIGURE 8.6 Tier approach for identification of agents with the potential to induce or influence autoimmune disease. Comprehensive examination of circulating autoantibodies can help to identify exposures that may pose a risk for development of autoimmunity. In addition, careful and comprehensive evaluation of pathological changes in target organs has the potential to be informative regarding changes known to be associated with autoimmunity. Together, findings from such a screening approach can not only identify agents with the autoimmunogenic potential, but can also identify likely targets of the misguided immune responses. The screen can identify the likely disease target and direct selection of appropriate autoimmune-prone animal models for further investigation and characterization of the effects of exposure to the agent. In addition to further characterizing the effects of autoimmunogenic agents, animal models can also be used to define the mechanisms and determinants of autoimmune disease. ANA, antinuclear antibodies; ENA, extractable nuclear antigens, SLE, systemic lupus erythromatosis; TSH, thyroid stimulating hormone, Tg, thyroglobuline; TPO, thyroid peroxidase; TSH-R, thyroid stimulating hormone receptor; TP, tyrosine phosphatase; GAD, glutamic acid decarboxylase; 21-OH, steroid 21-hydroxylase.

hazard identification.⁵⁴ Such an approach should start with an assessment of anti-self antibodies and irregularities in immunoglobulin production. Interpretation of humoral immune patterns in the context of immunopathological changes can help understand the potential for induction of systemic and/or organ-specific autoimmunity. An excellent resource for this effort is the wealth of information and biological samples that are obtained from 90-day to 2-year bioassays. The pathological findings from these studies could help identify potential organs and systems affected by the chemicals that increase the risk of developing autoimmune disease. In addition, serum banked from these studies (and in certain instances tissue) represents a valuable sample source to study the potential for autoimmune induction by the test chemical. Figure 8.6 provides an illustration of an example of a tiered approach to autoimmunity testing in immunotoxicology. Further research and development of testing strategies are critical for hazard identification and risk assessment for autoimmunity.

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REFERENCES

- Burleson, G. R. and Dean, J. H. 1995a. Immunotoxicology: Past, present, and future. In, *Methods in Immunotoxicology*, eds. G. R. Burleson, J. H. Dean, and A. E. Munson, Vol. 1, pp. 3–10. New York: Wiley-Liss.
- Koller, L. D., Exon, J. H., and Roan, J. G. 1976. Humoral antibody response in mice after single dose exposure to lead or cadmium. *Proceedings of the Society for Experimental Biology and Medicine*, 151:339–342.
- Vos, J. G. 1977. Immune suppression as related to toxicology. *CRC Critical Reviews in Toxicology*, 5:67–101.
- Gainer, J. H. 1977. Effects of heavy metals and of deficiency of zinc on mortality rates in mice infected with encephalomyocarditis virus. *American Journal of Veterinary Research*, 38:869–872.
- Dean, J. H., Padarathsingh, M. L., and Jerrells, T. R. 1979. Assessment of immunobiological effects induced by chemicals, drugs or food additives. I. Tier testing and screening approach. *Drug and Chemical Toxicology*, 2:5–17.
- Davies, G. E. 1983. Immunotoxicity: undesirable effects of inappropriate responses. *Immunology Today*, 4:1–2.
- Luster, M. I., Munson, A. E., Thomas, P. T., Holsapple, M. P., Fenters, J. D. et al. 1988. Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. *Fundamental and Applied Toxicology*, 10:2–19.
- Luster, M. I., Rosenthal, G. J., Cao, W., Thompson, M. B., Munson, A. E. et al. 1991. Experimental studies of the hematologic and immune system toxicity of nucleoside derivatives used against HIV infection. *International Journal of Immunopharmacology*, 13(Suppl 1): 99–107.
- Luster, M. I., Portier, C., Pait, D. G., White, K. L., Gennings, C. et al. 1992a. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundamental and Applied Toxicology*, 18:200–210.
- Luster, M. I., Pait, D. G., Portier, C., Rosenthal, G. J., Germolec, D. R. et al. 1992b. Qualitative and quantitative experimental models to aid in risk assessment for immunotoxicology. *Toxicology Letters*, 64–65 Spec: 71–78.
- Luster, M. I., Portier, C., Pait, D. G., Rosenthal, G. J., Germolec, D. R. et al. 1993. Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. *Fundamental and Applied Toxicology*, 21:71–82.
- Luster, M. I., Portier, C., Pait, D. G., Rosenthal, G. J., and Germolec, D. R. 1995. Immunotoxicology and risk assessment. In, *Methods in Immunotoxicology*, eds. G. R. Burleson, J. H. Dean, and A. E. Munson, Vol. 1, pp. 51–68. New York: Wiley-Liss.
- Germolec, D. R. 2004a. Sensitivity and predictivity in immunotoxicity testing: immune endpoints and disease resistance. *Toxicology Letters*, 149:109–114.
- Burleson, G. R. and Burleson, F. G. 2008a. Animal models of host resistance. In, *Immunotoxicology Strategies for Pharmaceutical Safety Assessment*, eds. D. J. Herzyk and J. L. Bussiere, pp. 167–177. Hoboken, NJ: John Wiley & Sons.
- Burleson, G. R. and Burleson, F. G. 2008b. Testing human biologicals in animal host resistance models. *Journal of Immunotoxicology*, 5:23–31.
- Burleson, F. G. and Burleson, G. R. 2010. Host resistance assays including bacterial challenge models. In, *Immunotoxicity Testing Methods and Protocols, Methods in Molecular Biology*, ed. R. R. Dietert, Vol. 598, pp. 97–108. New York: Springer.
- Burleson, G. R., Burleson, F. G., and Dietert, R. R. 2010. The cytotoxic T lymphocyte assay for evaluating cell-mediated immune function. In, *Immunotoxicity Testing Methods and Protocols, Methods in Molecular Biology*, ed. R. R. Dietert, Vol. 598, pp. 195–206. New York: Springer.
- Bretscher, P. A. 1983. Regulation of the class of immune response induced by antigen. I. Specific T cells switch the in vivo response from a cell-mediated to humoral mode. *Cellular Immunology*, 81:345–356.
- Jerne, N. K. and Nordin, A. A. 1963. Plaque formation in agar by single antibody-producing cells. *Science (New York, NY)*, 140:405.
- White, K. L., Sheth, C. M., and Peachee, V. L. 2007. Comparison of primary immune responses to SRBC and KLH in rodents. *Journal of Immunotoxicology*, 4:153–158.
- Burleson, G. R. 1995b. Influenza host resistance model for assessment of immunotoxicity, immunostimulation, and antiviral compounds. In, *Methods in Immunotoxicology*, eds. G. R. Burleson, J. H. Dean, and A. E. Munson, Vol. 2, pp. 181–202. New York: Wiley-Liss.
- Burleson, G. R. and Burleson, F. G. 2007. Influenza virus host resistance model. *Methods (San Diego, Calif.)*, 41:31–37.
- Germolec, D. R., Nyska, A., Kashon, M., Kuper, C. F., Portier, C. et al. 2004b. Extended histopathology in immunotoxicity testing; Interlaboratory validation studies. *Toxicological Sciences*, 78:107–115.
- Germolec, D. R., Kashon, M., Nyska, A., Kuper, C. F., Portier, C. et al. 2004c. The accuracy of extended histopathology to detect immunotoxic chemicals. *Toxicological Sciences*, 82:504–514.
- US EPA OPPTS 870.7800, Immunotoxicity, August 1998.
- USEPA 40 CFR Parts 9 and 158 Federal Register Notice/Vol. 72, No. 207 on October 26, 2007.
- ICH S8 Immunotoxicity Testing Guidance for Industry, April 2006.
- ICH S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, July 1997.

29. ICH S6 Addendum to Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, May 2012.
30. Duff, G. 2006. *Expert Scientific Group on Phase One Clinical Trials. Final Report*. Published by TSO (The Stationery Organization).
31. Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A. et al. 2006. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *The New England Journal of Medicine*, 355:1018–1028.
32. Horvath, C. J. and Milton, M. N. 2009. The TeGenero incident and the Duff Report conclusions: a series of unfortunate events or an avoidable event? *Toxicologic Pathology*, 37:372–383.
33. Gribble, E. J., Sivakumar, P. V., Ponce, R. A., and Hughes, S. D. 2007. Toxicity as a result of immunostimulation by biologics. *Expert Opinion on Drug Metabolism and Toxicology*, 3:209–234.
34. Coombs, R. R. and Gell, P. G. 1968. Classification of allergic reactions responsible for hypersensitivity and disease. In, *Clinical Aspects of Immunology*, eds. P. G. Gell and R. R. Coombs. The University of California, CA: Blackwell Scientific.
35. Norbury, K. and Thomas, P. T. 1990. *In Vivo Toxicity Testing: Principles, Procedures, and Practices*. New York: Academic Press.
36. Buehler, E. V. 1965. Delayed contact hypersensitivity in the guinea pig. *Archives of Dermatology*, 91:171–177.
37. Magnusson, B. and Kligman, A. M. 1970. *Allergic Contact Dermatitis in the Guinea Pig: Identification of Contact Allergens*. Springfield, IL: Charles C. Thomas.
38. Draize, J. H., Woodard, G., and Calvery, H. O. 1944. Methods for the study of irritation and toxicity of substances applied to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics*, 8:377–390.
39. Kimber, I., Mitchell, J. A., and Griffin, A. C. 1986. Development of a murine local lymph node assay for the determination of sensitizing potential. *Food and Chemical Toxicology*, 24:585–596.
40. Organization for Economic Co-operation and Development (OECD). 2010. *OECD Guideline for the Testing of Chemical Substances: Skin Sensitization: Local Lymph Node Assay*.
41. National Toxicology Program (NTP). 2009. *ICCVAM Performance Standards for the LLNA*.
42. ICCVAM Test Method Evaluation Report: The reduced murine local lymph node assay: An alternative test method using fewer animals to assess the allergic contact dermatitis potential of chemicals and products (NIH Publication No. 09-6439); and, Recommended performance standards: Murine local lymph node assay (NIH Publication No. 09-7357).
43. ICCVAM. 2011. *ICCVAM Test Method Evaluation Report: Usefulness and limitations of the murine local lymph node assay for potency categorization of chemicals using allergic contact dermatitis in humans*. NIH Publication No. 11-7709. Research Triangle Park, NC: National Institute of Environmental Health Sciences.
44. Murine Local Lymph Node Assay (LLNA) Performance Standards. European Centre for the Validation of Alternative Methods (ECVAM). October 2008.
45. National Toxicology Program (NTP). 2010. *ICCVAM test method evaluation report on using the murine local lymph node assay for testing pesticide formulations, metals, substances in aqueous solutions, and other products*.
46. Gad, S. C., Dunn, B. J., Dobbs, D. W., Reilly, C., and Walsh, R. D. 1986. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). *Toxicology and Applied Pharmacology*, 84:93–114.
47. Auttachoat, W., Germolec, D. R., Smith, M. J., White, K. L., and Guo, T. L. 2011. Contact sensitizing potential of annatto extract and its two primary color components, cis-bixin and norbixin, in female BALB/c mice. *Food and Chemical Toxicology*, 49:2638–2644.
48. Arts, J. H. E., De Jong, W. H., Van Triel, J. J., Schijf, M. A., De Klerk, A. et al. 2008. The respiratory local lymph node assay as a tool to study respiratory sensitizers. *Toxicological Sciences*, 106:423–434.
49. Johnson, V. J., Reynolds, J. S., Wang, W., Fluharty, K., and Yucesoy, B. 2011. Inhalation of ortho-phthalaldehyde vapor causes respiratory sensitization in mice. *Journal of Allergy*, 75:1052–1063.
50. Moscato, G., Vandenplas, O., Van Wijk, R. G., Malo, J.-L., Perfetti, L. et al. 2009. EAACI position paper on occupational rhinitis. *Respiratory Research*, 10:16.
51. Chan-Yeung, M. and Malo, J. L. 1994. Aetiological agents in occupational asthma. *European Respiratory Journal*, 7:346–371.
52. Germolec, D. R. 2005. Autoimmune diseases, animal models. In *Encyclopedic Reference of Immunotoxicology*, ed. H.-W. Vohr, pp. 75–79. Berlin, Germany: Springer-Verlag.
53. World Health Organization (WHO). 2006. *Environmental Health Criteria 236: Principles and Methods for Assessing Autoimmunity Associated with Exposure to Chemicals*.
54. Dietert, R. R., Dietert, J. M., and Gavalchin, J. 2010. Risk of autoimmune disease: challenges for immunotoxicity Testing. In, *Immunotoxicity Testing Methods and Protocols, Methods in Molecular Biology*, ed. R. R. Dietert, Vol. 598, pp. 39–51. New York: Springer.

9 Renal Toxicology

Renal Function Parameters for Adult Fischer-344, Sprague–Dawley, and Wistar Rats

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INTRODUCTION

This chapter summarizes renal function parameters that were evaluated in both sexes of three rat strains (Fischer-344, Sprague–Dawley, and Wistar). These parameters can be used to characterize the nephrotoxicity potential of test compounds. The data in this chapter are from control (untreated) animals. Resting renal function parameters were evaluated using a battery of noninvasive procedures in unanesthetized animals. The renal response to specific stress was also evaluated using extracellular volume expansion and a urine concentrating ability test. In a separate group of animals, a battery of invasive procedures in anesthetized animals was used to evaluate specific renal functions, for example, urine flow rate, glomerular filtration rate (GFR), fractional excretion of electrolytes, fractional reabsorption of water, and free water clearance.

METHODS

Fischer-344, Sprague–Dawley, and Wistar rats were received from Charles River Breeding Laboratory in Kingston, NY, at approximately 6 weeks of age. Rats were housed singly in suspended stainless steel cages with a wire mesh front and bottom. The animals were maintained at a mean temperature of 72°F ± 3°F, a humidity of 50% ± 5%, and a 12/12 h light/dark cycle. Water was supplied *ad libitum* by an automatic watering system, and Purina Certified Rodent Chow No. 5002 was also available *ad libitum*. Body weight and food consumption were recorded weekly (Tables 9.1 and 9.2).

RESTING RENAL FUNCTION

Each animal was placed in an individual metabolism cage for a 24-h urine sample collection. The following parameters were analyzed and recorded: 24-h urine volume, sodium, potassium, chloride, protein, glucose, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), osmolality, pH, and creatinine (Cr). Individual animal blood and serum samples were analyzed for blood urea nitrogen (BUN), protein, Cr, hematocrit (Hct), sodium, potassium, chloride, and osmolality (Tables 9.3 through 9.6).

EXTRACELLULAR VOLUME EXPANSION + 9-h URINE COLLECTION

The extracellular volume space was expanded by intraperitoneal (IP) injection of isotonic saline (at 2.5% of body weight [v/w]). Animals were then placed in individual metabolism units with food and water *ad libitum*. Urine was collected every 3 h for a 9-h period and analyzed for urine volume, sodium, potassium, chloride, osmolality, and Cr (Tables 9.7 and 9.8).

URINE CONCENTRATING ABILITY

To measure the urinary concentrating ability, the animals were placed in the individual metabolism units with food *ad libitum* but without drinking water. After the first 6-h sample was discarded, two consecutive 24-h urine samples were collected and analyzed for urine volume, sodium, potassium, chloride, osmolality, and Cr (Tables 9.9 and 9.10).

INVASIVE PARAMETERS

A separate group of animals was used to evaluate specific renal parameters best measured using invasive techniques. The experimental procedure was as follows: animals were anesthetized with 100 mg/kg IP injection of inactin*,¹ then placed on a heated surgical table. The animal was then tracheotomized with Clay Adams polyethylene tubing (PE) 240 to maintain an open airway. A catheter was inserted into the jugular vein (PE 50) and an intravenous (IV) infusion started. The infusion solution was isotonic saline with 2.5 μCi [^3H]inulin/mL infused at approximately 0.02 mL/100 g body weight/min. A PE 50 catheter was placed in the carotid artery for blood sample collection. An inulin bolus consisting of 25 μCi [^3H]inulin in 0.3 mL of isotonic saline was injected IV. The bladder was catheterized with PE 60 tubing through a suprapubic incision. The animal was then allowed to equilibrate for 30 min. During the next hour, the following measurements were made: urine flow rate and GFR [^3H]inulin clearance. Urine and plasma osmolality were determined along with urine/plasma inulin, sodium, potassium, and chloride ratios. Free water clearance and the fractional excretion of sodium, potassium, and chloride were also calculated. Kidneys were removed and weighed when each experiment was terminated (right and left kept separate).

The following measurements were performed (Tables 9.11 through 9.14):

Urine: volume, sodium, potassium, chloride, [^3H]inulin (liquid scintillation [^3H]inulin spectrophotometry), and osmolality

Serum: Hct, sodium, potassium, chloride, and osmolality

RENAL FUNCTION PARAMETERS

ABBREVIATIONS

GFR	glomerular filtration rate (mL/min)
Hct	hematocrit (%)
P_{Cl}	plasma chloride ($\mu\text{Eq/mL}$)
P_K	plasma potassium ($\mu\text{Eq/mL}$)
P_{Na}	plasma sodium ($\mu\text{Eq/mL}$)
P_{Osm}	plasma osmolality (mOsm/kg)
U_{Cl}	urine chloride ($\mu\text{Eq/mL}$)
U_K	urine potassium ($\mu\text{Eq/mL}$)
U_{Na}	urine sodium ($\mu\text{Eq/mL}$)
U_{Osm}	urine osmolality (mOsm/kg)
U/P_{Cl}	urine/plasma chloride ratio
U/P_{Inulin}	urine/plasma inulin ratio
U/P_K	urine/plasma potassium ratio
U/P_{Na}	urine/plasma sodium ratio
V	urine flow rate (mL/min)

TABLE 9.1

Mean (\pm SD) Body Weight (g) for Fischer-344, Sprague–Dawley, and Wistar Rats (8–25 Weeks of Age)^a

Rat Strains	Age (Weeks)															
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	24	25
F-344																
Males	224.8	239.3	252.7	268.6	280.0	289.0	294.4	303.9	312.3	321.5	323.6	328.9	328.3	328.1	340.6	343.1
SD	6.5	9.8	11.6	14.6	14.7	17.7	18.7	18.8	20.2	21.5	22.9	22.8	24.5	23.0	25.4	24.0
Sprague–Dawley																
Males	317.4	343.1	370.8	394.6	417.0	435.7	451.4	464.5	477.3	485.1	495.3	506.2	517.4	518.7	525.9	547.7
SD	16.6	16.9	21.2	23.4	27.4	26.8	28.7	32.4	33.1	35.7	38.1	40.6	42.7	45.3	49.8	46.9
Wistar																
Males	305.1	342.1	373.1	398.0	421.0	433.9	456.3	470.7	489.8	500.3	507.3	515.5	515.0	—	534.1	—
SD	10.6	8.5	10.0	9.0	9.1	11.6	12.3	12.6	16.9	13.6	14.1	17.2	20.3	—	21.4	—
F-344																
Females	143.8	146.6	151.9	159.9	163.1	166.6	166.0	169.3	173.4	177.7	176.3	179.5	180.5	179.4	184.6	183.4
SD	5.9	6.2	7.4	9.9	10.1	10.8	10.0	10.8	10.5	11.5	11.0	11.5	11.5	11.6	10.9	10.1
Sprague–Dawley																
Females	200.8	209.7	225.3	235.2	247.4	258.1	261.4	269.4	278.1	283.2	290.4	295.4	299.3	299.5	301.5	311.9
SD	14.0	19.5	17.8	21.0	24.5	25.2	26.9	30.4	31.6	30.4	31.5	32.0	32.6	31.6	33.9	35.6
Wistar																
Females	206.0	218.3	228.7	238.4	247.4	249.6	257.7	261.0	267.6	267.4	273.6	276.5	274.6	—	283.8	—
SD	16.2	17.5	17.9	17.9	20.5	22.0	23.3	24.3	23.7	25.7	24.7	23.8	24.0	—	24.9	—

^a n = 6/group; —, data unavailable; F-344, Fischer-344.

* Inactin, supplied by Byk Gulden Konstanz, West Germany, is the sodium salt of ethyl-(α -methyl-propyl)-malonylthio-urea.

TABLE 9.2**Mean (\pm SD) Weekly Food Consumption (g) for Fischer-344, Sprague–Dawley, and Wistar Rats (for 13 Weeks)**

Rat Strains	Week													
	0 ^a	1	2	3	4	5	6	7	8	9	10	11	12	13
F-344														
Males	128.3	141.0	118.4	119.1	119.7	116.9	117.9	116.7	120.6	119.5	117.9	112.2	111.9	105.9
SD	7.9	10.2	9.7	9.6	9.7	7.6	7.8	8.6	10.3	10.3	8.9	8.7	7.9	12.7
Sprague–Dawley														
Males	174.5	206.3	182.8	181.3	183.6	185.3	185.6	176.4	180.4	180.4	183.6	178.5	173.4	168.2
SD	7.17	12.7	12.9	11.7	13.9	17.7	18.2	19.0	16.5	21.6	23.8	19.8	18.5	13.9
Wistar														
Males	223.3	189.9	190.6	189.5	191.7	188.9	189.4	195.3	185.8	188.6	189.3	180.8	184.1	—
SD	7.5	5.7	9.1	9.0	7.8	10.1	9.5	12.6	17.8	12.4	12.7	14.6	14.7	—
F-344														
Females	82.9	95.4	85.5	84.2	82.8	77.8	82.6	82.6	82.5	83.4	83.1	84.2	79.3	71.1
SD	6.8	8.8	9.6	7.3	6.2	5.6	5.4	5.0	6.6	6.5	5.2	5.9	4.4	5.4
Sprague–Dawley														
Females	129.1	155.8	142.5	139.8	136.6	134.8	138.0	137.1	137.9	136.4	136.1	126.8	119.5	124.5
SD	12.5	18.7	16.4	15.7	9.1	10.9	16.8	23.0	21.5	12.7	11.0	12.9	11.9	12.4
Wistar														
Females	167.5	133.9	141.1	131.4	136.5	147.3	130.3	130.0	128.7	129.7	127.2	126.3	133.8	—
SD	18.0	15.2	20.1	10.7	10.9	24.2	18.0	13.0	7.7	8.5	11.2	8.9	8.3	—

^a Age at week 0 was approximately 8 weeks; n, 6/group; F-344, Fischer-344; —, data unavailable.**TABLE 9.3****24-h Mean Urinalysis Data with Standard Deviation and Standard Error of the Mean in Adult Male Rats: Fischer-344, Sprague–Dawley, and Wistar Rats**

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Volume (mL)	5.92	2.15	0.88	14.83	7.63	3.12	12.68	4.06	1.66
Volume (mL/100 g body weight)	1.78	0.556	0.227	2.824	1.339	0.547	2.453	0.761	0.311
Sodium (μ Eq/mL)	62.7	20.3	8.3	54.3	32.7	13.4	41.67	16.27	6.64
Potassium (μ Eq/mL)	197.67	32.87	13.42	168.0	75.2	30.7	146.0	37.5	15.3
Chloride (μ Eq/mL)	105.0	54.5	22.3	64.7	47.5	19.4	60.0	24.9	10.2
Protein (g/dL)	0.4833	0.0983	0.0401	0.5167	0.1941	0.0792	0.3667	0.0816	0.0333
Glucose (mg/dL)	7.33	17.96	7.33	0.00	0.00	0.00	0.00	0.00	0.00
ALP (IU)	154.2	54.4	22.2	87.1	53.7	21.9	141.4	43.4	17.8
LDH (IU)	3.83	9.39	3.83	34.17	83.69	34.17	0.00	0.00	0.00
Osmolality (mOsm/kg)	1312.3	210.5	86.0	1206	497	203	1197	325	133
pH	6.18	0.41	0.17	6.83	0.75	0.31	6.167	0.406	0.17
Cr (mg/dL)	144.2	22.8	9.3	142.0	61.9	25.3	165.7	60.7	24.8
Sodium/Cr (μ Eq/mg Cr)	43.2	124	5.07	35.78	7.88	3.22	25.37	7.55	3.08
Potassium/Cr (μ Eq/mg Cr)	137.2	11.6	4.72	117.29	15.55	6.35	91.10	16.1	6.59
Chloride/Cr (μ Eq/mg Cr)	70.3	35.3	14.40	39.5	25.0	10.20	36.9	14.7	5.99
Protein/Cr (g/mg Cr)	0.0039	0.00058	0.00024	0.0038	0.0012	0.00047	0.0023	0.0004	0.00016
Glucose/Cr (mg/mg Cr)	0.05	0.123	0.05	0	0	0	0	0	0

TABLE 9.4

24-h Mean Urinalysis Data with Standard Deviation and Standard Error of the Mean in Adult Female Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Volume (mL)	8.82	4.32	1.76	8.43	3.43	1.40	18.22	8.07	3.29
Volume (mL/100 g body weight)	4.93	2.41	0.98	2.839	1.119	0.457	6.48	2.46	1.00
Sodium (μEq/mL)	152.0	41.4	16.9	155.2	16.2	6.6	81.7	63.5	25.9
Potassium (μEq/mL)	304.0	91.2	36.8	324.2	50.8	20.7	179.7	97.9	40.0
Chloride (μEq/mL)	205.5	61.6	25.2	249.7	78.7	32.1	104.7	79.5	32.5
Protein (g/dL)	0.3333	0.0516	0.0211	0.4667	0.1211	0.0494	0.1833	0.0983	0.0401
Glucose (mg/dL)	9.3	14.5	5.9	8.33	13.05	5.33	0.00	0.00	0.00
ALP (IU)	25.22	11.14	4.55	16.1	11.1	4.5	32.0	24.9	10.2
LDH (IU)	0.00	0.00	0.00	13.83	19.02	7.76	2.50	6.12	2.50
Osmolality (mOsm/kg)	1764	520	212	2286	650	266	1083	428	175
pH	7.00	0.632	0.26	7.83	1.17	0.48	7.50	1.23	0.50
Cr (mg/dL)	91.8	27.8	11.4	161.5	54.2	22.1	71.50	23.98	9.79
Sodium/Cr (μEq/mg Cr)	169.9	27.7	11.32	105.5	34.6	14.13	120.9	85.0	34.72
Potassium/Cr (μEq/mg Cr)	334.6	27.7	11.32	213.5	47.5	19.39	264.1	129.3	52.77
Chloride/Cr (μEq/mg Cr)	225.6	24.6	10.06	162.8	43.8	17.90	149.9	106.8	43.62
Protein/Cr (g/mg Cr)	0.0040	0.0015	0.00062	0.0031	0.00086	0.00035	0.0027	0.0015	0.0006
Glucose/Cr (mg/mg Cr)	0.0817	0.1266	0.0517	0.0417	0.0646	0.0264	0	0	0

TABLE 9.5

Mean Serum Chemistry/Hematology Parameters with Standard Deviation and Standard Error of Mean in Adult Male Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
BUN (mg/dL)	17.333	0.848	0.346	16.15	2.08	0.85	20.63	2.14	0.87
Total protein (g/dL)	6.367	0.403	0.165	6.633	0.216	0.088	6.383	0.214	0.087
Cr (mg/dL)	0.830	0.172	0.070	0.860	0.145	0.059	1.015	0.154	0.0628
Osmolality (mOsm/kg H ₂ O)	316.50	11.33	4.62	307.50	3.45	1.41	315.33	5.05	2.06
Potassium (mEq/L)	4.45	0.35	0.141	4.98	0.48	0.20	5.02	0.33	0.14
Chloride (mEq/L)	101.50	1.87	0.76	101.50	2.17	0.89	97.67	1.63	0.67
Sodium (mEq/L)	148.17	1.33	0.54	146.67	2.25	0.92	151.50	2.17	0.89
HCT (%)	47.00	2.00	0.82	47.33	1.51	0.62	43.33	1.03	0.42

TABLE 9.6

Mean Serum Chemistry/Hematology Parameters with Standard Deviation and Standard Error of Mean in Adult Female Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
BUN (mg/dL)	19.18	2.39	0.98	17.58	2.08	0.85	19.10	1.04	0.43
Total protein (g/dL)	6.550	0.321	0.131	7.333	0.665	0.272	6.867	0.225	0.092
Cr (mg/dL)	0.653	0.124	0.051	0.713	0.086	0.035	0.707	0.100	0.041
Osmolality (mOsm/kg H ₂ O)	306.00	3.10	1.26	309.33	4.23	1.73	306.00	2.28	0.93
Potassium (mEq/L)	4.65	0.16	0.07	4.47	0.57	0.23	4.42	0.40	0.16
Chloride (mEq/L)	99.50	0.84	0.34	102.00	2.61	1.07	100.00	3.03	1.24
Sodium (mEq/L)	145.67	0.82	0.33	148.17	1.83	0.75	146.33	1.37	0.56
HCT (%)	44.50	1.87	0.76	42.50	2.88	1.18	42.83	2.32	0.95

TABLE 9.7

Extracellular Volume Expansion Test: Urinalysis with Standard Deviations and Standard Error of the Mean in Adult Male Rats (Fischer-344, Sprague–Dawley, and Wistar Rats)

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Volume (mL)									
+3 h	2.67	1.71	0.70	3.65	2.20	0.90	4.13	2.69	1.10
+6 h	3.23	1.64	0.67	7.43	2.10	0.86	9.83	3.81	1.55
+9 h	2.72	0.68	0.28	3.95	1.39	0.57	3.42	1.46	0.60
Total 9-h volume	8.62	2.33	0.95	15.03	3.51	1.43	17.38	7.22	2.95
Volume (mL/100 g body weight)									
+3 h	0.772	0.474	0.193	0.675	0.411	0.168	0.778	0.501	0.205
+6 h	0.946	0.490	0.200	1.366	0.325	0.133	1.832	0.662	0.270
+9 h	0.804	0.228	0.093	0.726	0.240	0.098	0.636	0.257	0.105
Total 9-h volume	2.52	0.645	0.263	2.767	0.567	0.231	3.245	1.283	0.524
Sodium (μEq/mL)									
+3 h	150.3	36.2	14.8	145.0	50.4	20.6	89.0	37.2	15.2
+6 h	137.7	45.1	18.4	78.3	18.9	7.7	69.7	17.7	7.2
+9 h	130.7	12.5	5.1	100.7	38.1	15.5	106.3	14.1	5.8
Potassium (μEq/mL)									
+3 h	200.7	50.3	20.5	201.0	72.8	29.7	161.7	94.2	38.5
+6 h	123.3	66.5	27.1	102.0	21.9	8.9	81.3	22.4	9.1
+9 h	100.3	16.7	6.8	83.3	20.3	8.3	72.3	13.7	5.6
Chloride (μEq/mL)									
+3 h	266.7	66.9	27.3	238.2	98.0	40.0	177.0	74.1	30.2
+6 h	203.0	91.3	37.3	147.2	26.9	11.0	111.0	35.2	14.4
+9 h	158.8	20.3	8.3	105.7	45.3	18.5	113.3	18.3	7.5
Osmolality (mOsm/kg)									
+3 h	1235	306	125	1322	460	186	1055	613	250
+6 h	931	421	172	662	134	55	549	139	57
+9 h	876	127	52	692	83	34	662	195	80
Cr (mg/dL)									
+3 h	59.8	17.2	7.0	84.8	24.7	10.1	79.5	61.1	25.0
+6 h	58.0	26.6	10.9	41.3	12.2	5.0	38.3	14.6	5.9
+9 h	59.0	11.6	4.7	69.3	16.1	6.6	57.5	20.2	8.2
Sodium/Cr (μEq/mg Cr)									
+3 h	260.2	62.2	25.38	173.5	35.9	14.65	148.0	69.7	28.5
+6 h	253.3	63.3	29.86	205.7	81.7	33.36	196.2	73.0	29.80
+9 h	229.6	54.5	22.24	156.0	70.4	28.74	198.2	54.9	22.39
Potassium/Cr (μEq/mg Cr)									
+3 h	339.1	33.9	13.86	234.3	27.3	11.15	218.05	39.5	16.1
+6 h	213.8	46.5	18.96	255.0	54.3	22.15	222.	56.3	22.97
+9 h	172.4	25.7	10.51	123.9	34.8	14.22	131.5	24.2	9.88
Chloride/Cr (μEq/mg Cr)									
+3 h	452.7	53.9	22.02	278.1	46.8	19.09	257.8	68.4	27.9
+6 h	359.6	62.3	25.42	374.5	100.8	41.14	305.7	110.6	45.14
+9 h	275.3	50.6	20.55	162.8	79.4	32.40	206.5	35.4	14.47

TABLE 9.8

Extracellular Volume Expansion Test: Urinalysis with Standard Deviations and Standard Error of the Mean in Adult Female Rats (Fischer-344, Sprague–Dawley, and Wistar Rats)

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Volume (mL)									
+3 h	2.82	1.40	0.57	1.72	1.04	0.43	5.00	4.16	1.70
+6 h	2.32	0.90	0.37	6.53	2.29	0.93	10.15	2.21	0.90
+9 h	1.88	0.40	0.16	2.32	0.63	0.26	3.22	1.37	0.56
Total 9-h volume	7.02	1.78	0.73	10.57	3.15	1.29	18.37	4.34	1.77
Volume (mL/100 g body weight)									
+3 h	1.549	0.852	0.348	0.592	0.401	0.164	1.847	1.556	0.635
+6 h	1.267	0.517	0.211	2.218	0.822	0.335	3.598	0.803	0.328
+9 h	1.021	0.205	0.064	0.785	0.256	0.104	1.140	0.492	0.201
Total 9-h volume	3.836	1.145	0.467	3.595	1.251	0.511	6.585	1.96	0.80
Sodium (μEq/mL)									
+3 h	126.3	17.7	7.2	136.3	48.2	19.7	60.8	28.2	12.6
+6 h	145.3	59.2	24.2	111.7	18.7	7.6	105.0	11.8	4.8
+9 h	126.3	54.9	22.4	158.0	49.8	20.4	114.0	27.4	11.2
Potassium (μEq/mL)									
+3 h	168.7	41.4	16.9	166.0	80.0	32.7	70.8	45.6	20.4
+6 h	119.3	47.1	19.2	102.7	57.4	23.4	54.7	15.2	6.2
+9 h	106.3	44.6	18.2	116.0	62.2	25.4	68.3	18.6	7.6
Chloride (μEq/mL)									
+3 h	204.2	33.9	13.9	225.7	111.7	45.6	98.2	72.5	32.4
+6 h	182.3	63.0	25.7	174.8	43.0	17.5	123.3	20.5	8.4
+9 h	149.7	53.1	21.7	142.8	50.1	20.5	95.5	46.5	19.0
Osmolality (mOsm/kg)									
+3 h	1055	281	115	1319	694	283	658	523	234
+6 h	894	353	144	723	370	151	445	96	39
+9 h	845	298	122	885	455	186	546	116	47
Cr (mg/dL)									
+3 h	40.0	15.5	6.3	78.5	48.6	19.8	43.4	45.2	20.2
+6 h	33.2	10.8	4.4	33.3	25.1	10.2	23.3	15.7	6.4
+9 h	33.8	5.6	2.3	55.3	29.3	12.0	40.7	7.7	3.2
Sodium/Cr (μEq/mg Cr)									
+3 h	340.4	81.9	33.44	211.4	104.9	42.83	213.4	121.4	54.28
+6 h	432.1	72.0	29.38	423.3	158.9	64.86	553.3	195.7	79.91
+9 h	378.0	143.0	58.36	326.4	128.9	52.62	287.8	75.0	30.63
Potassium/Cr (μEq/mg Cr)									
+3 h	436.5	60.5	24.68	227.8	82.7	33.76	198.3	69.1	30.91
+6 h	352.5	36.2	14.76	328.1	62.0	25.30	269.5	78.9	32.20
+9 h	310.7	92.8	37.87	213.6	46.7	19.06	174.8	59.7	24.37
Chloride/Cr (μEq/mg Cr)									
+3 h	546.0	120.6	49.24	318.2	151.3	61.78	266.1	223.2	99.81
+6 h	550.2	47.8	19.52	625.8	167.1	68.22	628.8	196.0	80.03
+9 h	446.0	136.0	55.54	299.2	137.8	56.25	235.8	112.7	46.00

TABLE 9.9
48-h Urine Concentrating Test: Urinalysis with Standard Deviations and Standard Error of the Mean in Adult Male Rats (Fischer-344, Sprague–Dawley, and Wistar Rats)

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Volume (mL)									
+24 h	3.80	1.45	0.59	6.18	1.54	0.63	7.32	3.06	1.25
+49 h	1.62	0.48	0.19	2.13	0.27	0.11	2.95	1.51	0.62
Total	5.417	1.69	0.688	8.32	1.33	0.54	10.27	4.36	1.78
Volume (mL/100 g body weight)									
+24 h	1.09	0.362	0.148	1.125	0.253	0.103	1.318	0.562	0.230
+48 h	0.473	0.144	0.059	0.394	0.075	0.030	0.534	0.284	0.116
Total	1.563	0.427	0.174	1.519	0.217	0.088	1.852	0.811	0.331
Sodium (μEq/mL)									
+24 h	90.2	42.6	17.4	81.0	30.9	12.6	79.0	39.7	16.2
+48 h	196.2	55.1	22.5	144.3	41.8	17.1	132.7	38.0	15.5
Potassium (μEq/mL)									
+24 h	294.0	79.6	32.5	237.0	55.9	22.8	229.0	79.6	32.5
+48 h	453.8	97.2	39.7	292.7	44.5	18.2	281.0	83.5	34.1
Chloride (μEq/mL)									
+24 h	143.0	75.5	30.8	75.0	24.5	10.0	79.7	34.4	14.1
+48 h	234.2	80.1	32.7	106.7	65.1	26.6	91.2	51.0	20.8
Osmolality (mOsm/kg)									
+24 h	2171	472	193	1839	310	127	1937	591	241
+48 h	3618	502	205	3249	268	109	3251	550	225
Cr (mg/dL)									
+24 h	224.7	40.8	16.6	204.0	37.8	15.4	295.7	82.2	33.6
+48 h	399.7	37.6	15.3	599.5	48.8	19.9	536.2	149.1	60.9
Sodium/Cr (μEq/mg Cr)									
+24 h	39.04	13.08	5.34	39.10	11.36	4.64	29.29	17.12	6.99
+48 h	48.43	9.93	4.05	24.26	7.57	3.09	24.97	5.59	2.28
Potassium/Cr (μEq/mg Cr)									
+24 h	130.5	22.4	9.16	116.46	20.90	8.53	77.48	14.24	5.82
+48 h	113.8	21.5	8.79	49.10	8.51	3.47	52.72	10.52	4.29
Chloride/Cr (μEq/mg Cr)									
+24 h	60.4	25.7	10.49	37.06	11.66	4.76	28.99	14.36	5.86
+48 h	58.0	16.0	6.51	18.39	11.50	4.69	16.61	8.55	3.49

TABLE 9.10

48-h Urine Concentrating Test: Urinalysis with Standard Deviations and Standard Error of the Mean in Adult Female Rats (Fischer-344, Sprague–Dawley, and Wistar Rats)

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Volume (mL)									
+24 h	4.48	0.35	0.15	6.70	1.80	0.73	6.63	1.18	0.48
+48 h	1.16	0.82	0.33	2.27	0.52	0.21	2.48	0.28	0.11
Total	5.65	0.89	0.363	8.97	2.05	0.84	9.12	1.40	0.57
Volume (mL/100 g body weight)									
+24 h	2.45	0.247	0.101	2.13	0.456	0.186	2.257	0.321	0.131
+48 h	0.632	0.44	0.179	0.732	0.170	0.069	0.849	0.088	0.036
Total	3.08	0.48	0.20	2.87	0.524	0.214	3.11	0.376	0.154
Sodium (μEq/mL)									
+24 h	248.0	14.3	5.8	250.0	38.7	15.8	214.5	90.0	36.8
+48 h	233.3	40.5	16.5	266.0	51.0	20.8	210.2	68.1	27.8
Potassium (μEq/mL)									
+24 h	510.7	18.4	7.5	445.0	97.3	39.7	447.0	132.1	53.9
+48 h	638.7	48.2	19.7	595.3	110.1	44.9	514.8	77.3	31.6
Chloride (μEq/mL)									
+24 h	343.5	28.5	11.6	280.0	67.5	27.6	268.3	122.1	49.9
+48 h	296.3	49.1	20.1	284.5	62.4	25.5	284.0	125.4	51.2
Osmolality (mOsm/kg)									
+24 h	2974	64	26	2750	412	168	2625	577	236
+48 h	4073	279	114	4126	688	281	3908	687	280
Cr (mg/dL)									
+24 h	223.8	12.1	4.9	160.0	20.6	8.4	192.3	29.1	11.9
+48 h	339.7	33.2	13.6	345.5	50.8	20.7	337.7	39.0	15.9
Sodium/Cr (μEq/mg Cr)									
+24 h	111.18	10.36	4.23	156.5	16.8	6.84	109.6	45.6	18.64
+48 h	69.84	17.85	7.29	77.60	13.29	5.43	63.65	24.52	10.01
Potassium Cr (μEq/mg Cr)									
+24 h	228.7	14.4	5.89	277.4	43.1	17.59	230.5	57.4	23.42
+48 h	189.4	22.0	8.97	173.2	26.2	10.68	153.9	27.3	11.13
Chloride/Cr (μEq/mg Cr)									
+24 h	153.7	13.2	5.38	173.8	26.9	10.98	137.8	66.0	26.94
+48 h	88.3	20.0	8.15	83.3	17.4	7.11	85.3	38.9	15.90

TABLE 9.11

Kidney Weight Data with Standard Deviations and Standard Error of the Mean in Adult Male Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Left kidney weight (g)	1.230	0.067	0.027	1.808	0.247	0.101	1.860	0.172	0.070
Right kidney weight (g)	1.223	0.092	0.037	1.870	0.310	0.126	1.910	0.157	0.064
Total kidney weight (g)	2.453	0.153	0.062	3.68	0.551	0.225	3.77	0.327	0.133
Terminal body weight (g)	356.9	23.9	9.8	565.0	50.8	20.7	556.5	21.6	8.8
Kidney/body weight ratio $\times 1000$	6.88	0.337	0.138	6.49	0.52	0.21	6.77	0.44	0.18

TABLE 9.12

Kidney Weight Data with Standard Deviations and Standard Error of the Mean in Adult Female Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
Left kidney weight (g)	0.712	0.039	0.016	1.155	0.142	0.058	1.045	0.145	0.059
Right kidney weight (g)	0.712	0.028	0.011	1.188	0.166	0.068	1.065	0.129	0.052
Total kidney weight (g)	1.42	0.060	0.024	2.34	0.301	0.123	2.11	0.272	0.111
Terminal body weight (g)	189.8	9.6	3.9	322.5	38.1	15.6	293.7	28.1	11.5
Kidney/body weight ratio $\times 1000$	7.51	0.28	0.11	7.32	0.97	0.40	7.18	0.57	0.23

TABLE 9.13

Renal Function Test Parameters with Standard Deviations and Standard Error of the Mean in Adult Male Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
\dot{V} (mL/min)	0.00366	0.00181	0.00064	0.009012	0.00525	0.00186	0.003837	0.002206	0.000780
HCT (%)	46.56	1.86	0.66	45.94	1.95	0.69	45.81	1.49	0.53
U_{Na} (μ Eq/mL)	113.3	57	20.2	144.5	64.4	22.8	89.4	65.6	24.8
U_K (μ Eq/mL)	255	120.6	42.6	322	206	73	273.1	94.6	35.7
U_{Cl} (μ Eq/mL)	231.7	150.8	53.3	236.4	143.7	50.8	174.7	153.8	58.1
P_{Na} (μ Eq/mL)	147.56	6.06	2.14	143.63	4.17	1.48	143.63	1.30	0.46
P_K (μ Eq/mL)	4.25	0.342	0.121	4.375	0.324	0.115	4.612	0.285	0.101
P_{Cl} (μ Eq/mL)	101.56	4.94	1.75	105.13	2.10	0.74	102.88	1.356	0.479
U_{Osm} (mOsm/kg)	1753	834	295	1747	598	226	1503	596	243
P_{Osm} (mOsm/kg)	303.8	39.6	14.0	308.6	31.1	11.0	306.8	18.1	6.4
GFR (mL/min)	1.844	0.912	0.323	3.62	1.94	0.69	3.322	0.880	0.311
U/P_{Inulin}	566	284	100	530	204	72	1129	569	210
U/P_{Na}	0.775	0.396	0.140	1.011	0.456	0.161	0.617	0.460	0.174
U/P_K	61.3	29.6	10.5	75.7	50.4	17.8	60.5	21.3	8.0
U/P_{Cl}	2.32	1.60	0.57	2.234	1.338	0.473	1.689	1.474	0.577
C_{H_2O} (mL/min)	0.01842	0.01505	0.00532	−0.0430	0.0166	0.0063	−0.0209	0.0158	0.0065
FR_{H_2O} (%)	99.77	0.107	0.038	99.75	0.1641	0.0580	99.90	0.0475	0.0168
FE_{Na} (%)	0.196	0.161	0.057	0.3084	0.3661	0.1294	0.0856	0.0874	0.0330
FE_K (%)	14.22	9.84	3.48	16.28	8.93	3.16	7.52	4.80	1.82
FE_{Cl} (%)	0.504	0.389	0.137	0.682	0.748	0.264	0.2324	0.2751	0.1040
$U_{Na} \times \dot{V}$ (μ Eq/min)	0.499	0.530	0.187	1.41	1.399	0.495	0.470	0.515	0.195
$U_K \times \dot{V}$ (μ Eq/min)	0.966	0.596	0.211	2.899	1.916	0.677	1.282	0.994	0.376
$U_{Cl} \times \dot{V}$ (μ Eq/min)	0.989	1.013	0.358	2.604	2.473	0.874	0.949	1.194	0.451
\dot{V} (mL/min/g kidney weight)	1.805	1.014	0.359	2.649	1.758	0.622	1.101	0.575	0.203
U_{Osm} (mOsm/kg/g kidney weight)	856	406	144	496	150	57	435	171	70
GFR (mL/min/g kidney weight)	0.907	0.461	0.163	1.035	0.567	0.200	0.972	0.198	0.070
C_{H_2O} (mL/min/g kidney weight)	−0.0092	0.0081	0.0029	−0.01235	0.00493	0.00187	−0.00593	0.00443	0.00181
FR_{H_2O} (%/g kidney weight)	48.64	5.32	1.88	28.34	3.21	1.13	29.77	3.42	1.21
FE_{Na} (%/g kidney weight)	0.0963	0.0840	0.0297	0.0902	0.1074	0.03796	0.02470	0.02609	0.00986
FE_K (%/g kidney weight)	6.84	4.72	1.67	4.707	2.706	0.957	2.174	1.340	0.506
FE_{Cl} (%/g kidney weight)	0.284	0.210	0.074	0.2046	0.2278	0.0805	0.0660	0.0798	0.0302
$U_{Na} \times \dot{V}$ (μ Eq/min/g kidney weight)	0.2505	0.2875	0.1017	0.4152	0.4237	0.1498	0.1343	0.1519	0.0574
$U_K \times \dot{V}$ (μ Eq/min/g kidney weight)	0.472	0.313	0.111	0.837	0.566	0.20	0.363	0.258	0.098
$U_{Cl} \times \dot{V}$ (μ Eq/min/g kidney weight)	0.495	0.544	0.192	0.784	0.795	0.281	0.2673	0.3421	0.1293
Left kidney weight (g)	1.0329	0.1043	0.0369	1.717	0.155	0.055	1.630	0.231	0.082
Right kidney weight (g)	1.0386	0.1152	0.0407	1.839	0.225	0.080	1.766	0.232	0.082
Total kidney weight (g)	2.0715	0.2133	0.0754	3.556	0.370	0.131	3.396	0.406	0.144
Body weight (g)	326.0	30.5	10.8	544.2	26.9	9.5	551.9	48.8	17.2
Kidney/body weight ratio $\times 1000$	6.349	0.137	0.048	6.524	0.464	0.164	6.156	0.523	0.185

TABLE 9.14

Renal Function Test Parameters with Standard Deviations and Standard Error of the Mean in Adult Female Rats: Fischer-344, Sprague–Dawley, and Wistar Rats

Parameters	Strain								
	F-344			Sprague–Dawley			Wistar		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
\dot{V} (mL/min)	0.00325	0.00208	0.00078	0.00344	0.00248	0.00088	0.003414	0.003262	0.001233
HCT (%)	43.56	6.30	2.23	44.13	4.49	1.59	44.71	3.05	1.15
U_{Na} (μ Eq/mL)	171.9	60.6	21.4	127	45.6	17.2	105.9	65.2	24.7
U_K (μ Eq/mL)	153	133	47	292.6	160.7	60.7	177.3	94.2	35.6
U_{Cl} (μ Eq/mL)	207.5	153.6	54.3	186.6	107.3	40.6	105.9	102.9	38.9
P_{Na} (μ Eq/mL)	144.50	5.71	2.02	148.75	6.32	2.23	147.43	4.04	1.53
P_K (μ Eq/mL)	3.537	0.334	0.118	4.075	0.282	0.100	4.329	0.160	0.061
P_{Cl} (μ Eq/mL)	101.875	1.356	0.479	103.13	1.25	0.44	107.29	2.812	1.063
U_{Osm} (mOsm/kg)	1025	546	223	1411	660	295	1150	536	219
P_{Osm} (mOsm/kg)	874.1	1485.9	525.4	403.5	65.5	23.1	316.3	18.2	6.9
GFR (mL/min)	0.587	0.245	0.087	1.219	0.739	0.261	1.483	0.463	0.175
U/P_{Inulin}	207	122	43	434	343	121	757	583	220
U/P_{Na}	1.19	0.414	0.146	0.843	0.280	0.106	0.716	0.437	0.165
U/P_K	43.4	38.5	13.6	72.5	40.5	15.3	40.7	20.7	7.8
U/P_{Cl}	2.031	1.506	0.532	1.816	1.054	0.398	1.135	1.001	0.409
C_{H_2O} (mL/min)	0.00565	0.00674	0.00275	−0.0131	0.01341	0.0060	−0.0084	0.00608	0.00248
FR_{H_2O} (%)	96.93	1.6685	0.5899	97.045	7.114	2.5143	99.78	0.1571	0.0594
FE_{Na} (%)	1.53	2.368	0.835	0.478	0.7099	0.2683	0.2071	0.2844	0.1075
FE_K (%)	32.67	37.31	13.19	23.21	18.66	6.30	8.42	6.48	2.45
FE_{Cl} (%)	1.451	1.033	0.365	0.648	0.499	0.189	0.3783	0.5501	0.2283
$U_{Na} \times \dot{V}$ (μ Eq/min)	0.646	0.352	0.124	0.566	0.550	0.208	0.533	0.854	0.323
$U_K \times \dot{V}$ (μ Eq/min)	0.422	0.375	0.132	1.29	1.444	0.546	0.623	0.587	0.222
$U_{Cl} \times \dot{V}$ (μ Eq/min)	0.657	0.547	0.193	0.834	0.066	0.327	0.728	1.150	0.469
\dot{V} (mL/min/g kidney weight)	2.39	1.37	0.48	1.792	1.335	0.472	1.843	1.718	0.649
U_{Osm} (mOsm/kg/g kidney weight)	801	492	201	744	339	152	617	260	106
GFR (mL/min/g kidney weight)	0.440	0.200	0.071	0.684	0.404	0.143	0.814	0.215	0.081
C_{H_2O} (mL/min/g kidney weight)	−0.00467	0.00560	0.00228	−0.0070	0.00684	0.00906	−0.00448	0.00816	0.00129
FR_{H_2O} (%/g kidney weight)	74.03	6.12	2.17	50.30	12.81	4.53	55.58	4.52	1.71
FE_{Na} (%/g kidney weight)	1.140	1.742	0.616	0.2149	0.2611	0.0987	0.1120	0.1514	0.0572
FE_K (%/g kidney weight)	24.96	27.87	9.85	11.69	7.65	2.89	4.54	3.37	1.27
FE_{Cl} (%/g kidney weight)	1.105	0.801	0.283	0.3259	0.2316	0.0875	0.2016	0.2983	0.1218
$U_{Na} \times \dot{V}$ (μ Eq/min/g kidney weight)	0.416	0.278	0.098	0.293	0.283	0.107	0.2858	0.4553	0.1721
$U_K \times \dot{V}$ (μ Eq/min/g kidney weight)	0.334	0.318	0.112	0.684	0.746	0.282	0.334	0.307	0.116
$U_{Cl} \times \dot{V}$ (μ Eq/min/g kidney weight)	0.511	0.465	0.164	0.447	0.457	0.173	0.3875	0.6141	0.2507
Left kidney weight (g)	0.6566	0.0542	0.0192	0.9685	0.1990	0.0704	0.8624	0.0605	0.0229
Right kidney weight (g)	0.6695	0.0520	0.0184	1.055	0.235	0.083	0.9426	0.0834	0.0315
Total kidney weight (g)	1.3261	0.1026	0.0363	2.023	0.432	0.153	1.805	0.138	0.052
Body weight	183.37	9.03	3.19	278.7	36.0	12.7	273.8	20.2	7.1
Kidney/body weight ratio $\times 1000$	7.235	0.463	0.164	7.279	1.352	0.478	6.576	0.474	0.179

REFERENCE

1. AlliedSignal Inc., AlliedSignal Internal Report No. MA179811, October 3, 1983.

10 Developmental and Reproductive Toxicity

Robert M. Parker, PhD, DABT

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DEVELOPMENTAL TOXICOLOGY

INTRODUCTION

The purposes of DART studies are to determine whether a test substance has the potential to cause adverse effects on the male and female reproductive system or the developing conceptus, and to determine a developmental and/or reproductive no-observable-effect-level (NOEL) or no-observable-adverse-effect-level (NOAEL) for the test substance. The developmental or reproductive NOEL is the highest treatment dosage tested that shows no developmental or reproductive effects, respectively, while the developmental or reproductive NOAEL is the highest treatment level tested that shows no *adverse* developmental or reproductive effects. It should be noted that DART study designs were not developed to cover every male and female reproductive parameter, developmental parameter, specific target organ, or mechanism of toxicity. For example, traditional DART studies do not provide information on maternal or paternal endocrine status, the placental

transfer or milk transfer of an agent or its metabolites, placental pathology, or reproductive senescence.

DEVELOPMENTAL TOXICITY STUDY DESIGNS

DART study guidelines have been promulgated by the FDA,^{1–6} the EPA,^{7–10} the MAFF,^{11,12} Canada,^{13,14} Great Britain,¹⁵ WHO,¹⁶ and the OECD.^{17–21} The ICH^{6,7,22} has been concerned with unifying the study guideline requirements for pharmaceutical safety assessment for the European Union, Japan, and the FDA. Guideline compliant reproductive and fertility studies must be performed in accordance with associated Good Laboratory Practice Regulations,^{23–25} and biosafety²⁶ and animal welfare guidelines.^{27,28}

According to the ICH S5 (R2), Guideline for Industry, Detection of Toxicity to Reproduction for Medicinal Products,²⁹ a testing regimen should be selected that would “allow exposure of mature adults and all stages of development from conception to sexual maturity. To allow detection of immediate and latent effects of exposure, observations should be continued through one complete life cycle, that is, from conception

in one generation through conception in the following generation.” The document further suggests that an integrated test sequence can be subdivided into the following stages:

- Stage A: Premating to conception (adult male and female reproductive functions, development and maturation of gametes, mating behavior, fertilization).
- Stage B: Conception to implantation (adult female reproductive functions, preimplantation development, implantation).
- Stage C: Implantation to closure of the hard palate (adult female reproductive functions, embryonic development, major organ formation).
- Stage D: Closure of the hard palate to the end of pregnancy (adult female reproductive functions, fetal development and growth, organ development and growth).
- Stage E: Birth to weaning (adult female reproductive functions, neonate adaptation to extrauterine life, preweaning development and growth).
- Stage F: Weaning to sexual maturity (postweaning development and growth, adaptation to independent life, attainment of full sexual function).

Nevertheless, in most cases, these six stages are assessed by means of just three studies:

1. The fertility and reproductive toxicity study (“Segment I”: Stages A to B)
2. The prenatal developmental toxicity study (“Segment II”: Stages C to D, Figure 10.2)
3. The prenatal and postnatal study (“Segment III”: Stages C to F, Figure 10.3)

The fertility study (also known as ICH 4.1.1 study) covers the period of premating, cohabitation and mating, and early

pregnancy through implantation (gestation day [GD] 6 in the rat) (Figure 10.1). Fertility data can be analyzed by using the formulas listed in Table 10.1. For further descriptions of each parameter, see Parker.²⁰

The teratology study (also known as teratology study, embryo–fetal toxicity study, or ICH 4.1.3 study) covers pregnancy from implantation through major organogenesis (closure of the hard palate; GD 15 in the rat) up to the day before delivery (GD 20/21) (Figure 10.2), while the prenatal and postnatal studies (also known as ICH 4.1.2. study) cover late pregnancy and postnatal development (usually until weaning at lactation/postnatal day [LD or PND] 21) (Figure 10.3).

Combination studies, such as Segment I/II, Segment I/III, Segment II/III, and Segment I/II/III studies are acceptable as long as all required parameters and minimum study requirements for each segment are met. Other examples of acceptable combinatory studies are the 90-day repeated dose subchronic study with a one-generation reproduction study (Figure 10.4) and the US EPA and OECD combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (Figure 10.5). Multigenerational studies (studies covering two to three generations) are required for chemicals that pose a low-level but chronic exposure risk in humans (Figure 10.6). Recently, an extended one-generation toxicity study design has been promulgated by the OECD (Figure 10.7).

In addition, in recent years increased emphasis has been placed on direct toxicity studies in juvenile animals^{30,31} and developmental immunotoxicity.³² The nonhuman primate developmental and reproductive toxicity,^{33,34} and developmental immunotoxicology³⁵ study designs can be found in the cited studies and will not be discussed here. The juvenile toxicity study designs will be discussed in a subsequent chapter.

TABLE 10.1
Fertility and Reproductive Indices Used in Single and Multigeneration Studies

Index	Derivation
Mating	$= \frac{\text{No. confirmed copulations}}{\text{No. of estrous cycles required}} \times 100$
Male fertility	$= \frac{\text{No. males impregnating females}}{\text{No. males exposed to fertile, nonpregnant females}} \times 100$
Female fertility	$= \frac{\text{No. of females confirmed pregnant}}{\text{No. of females housed with fertile male}} \times 100$
Female fecundity	$= \frac{\text{No. of females confirmed pregnant}}{\text{No. of confirmed copulations}} \times 100$
Implantation	$= \frac{\text{No. of implantations}}{\text{No. of pregnant females}} \times 100$
Preimplantation loss	$= \frac{\text{Corpora lutea} - \text{No. of implants}}{\text{No. of Corpora lutea}} \times 100$
Parturition incidence	$= \frac{\text{No. of females giving birth}}{\text{No. of females confirmed pregnant}} \times 100$

TABLE 10.1 (continued)
Fertility and Reproductive Indices Used in Single and Multigeneration Studies

Index	Derivation
Live litter size	$= \frac{\text{No. of litters with live pups}}{\text{No. of females confirmed pregnant}} \times 100$
Live Birth	$= \frac{\text{No. viable pups born/litter}}{\text{No. pups born/litter}} \times 100$
Viability	$= \frac{\text{No. of viable pups born}}{\text{No. of dead pups born}} \times 100$
Survival	$= \frac{\text{No. of pups viable on day 1}}{\text{No. of viable pups born}} \times 100$
Pup death (day 1–4)	$= \frac{\text{No. of pups dying, postnatal days 1–4}}{\text{No. of viable pups born}} \times 100$
Pup death (days 5–21)	$= \frac{\text{No. of pups dying, postnatal days 5–21}}{\text{No. of viable pups born}} \times 100$
Sex ratio (at birth)	$= \frac{\text{No. of male offspring}}{\text{No. of female offspring}} \times 100$
Sex ratio (day 4) (day 21)	$= \frac{\text{No. of male offspring}}{\text{No. of female offspring}} \times 100$

Source: Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5, p. 98. With permission.

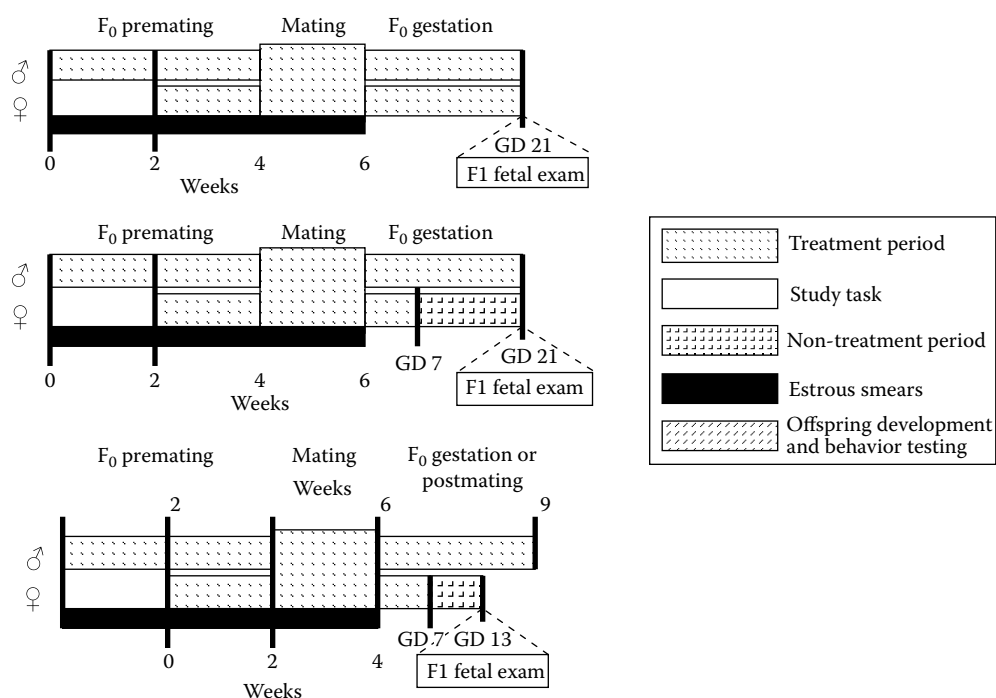


FIGURE 10.1 Fertility study schematics (FDA Segment I and ICH S5 [R2]).

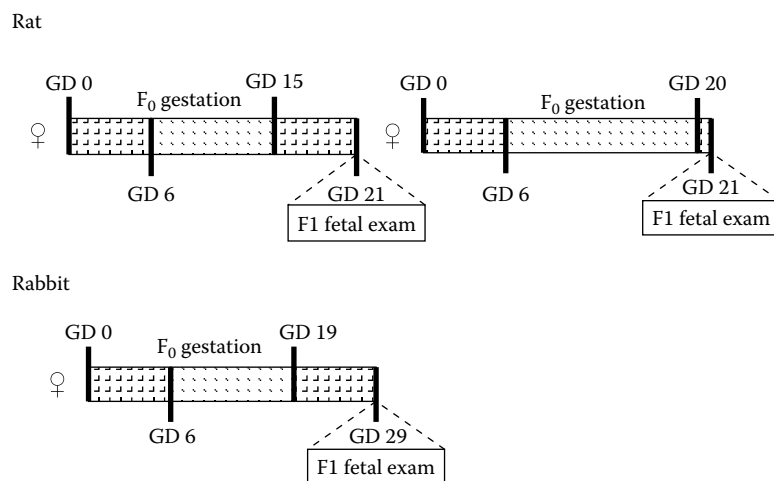


FIGURE 10.2 Developmental toxicity study schematics (FDA Segment II Study and ICH, OPPTS 870.3700 and OECD 414).

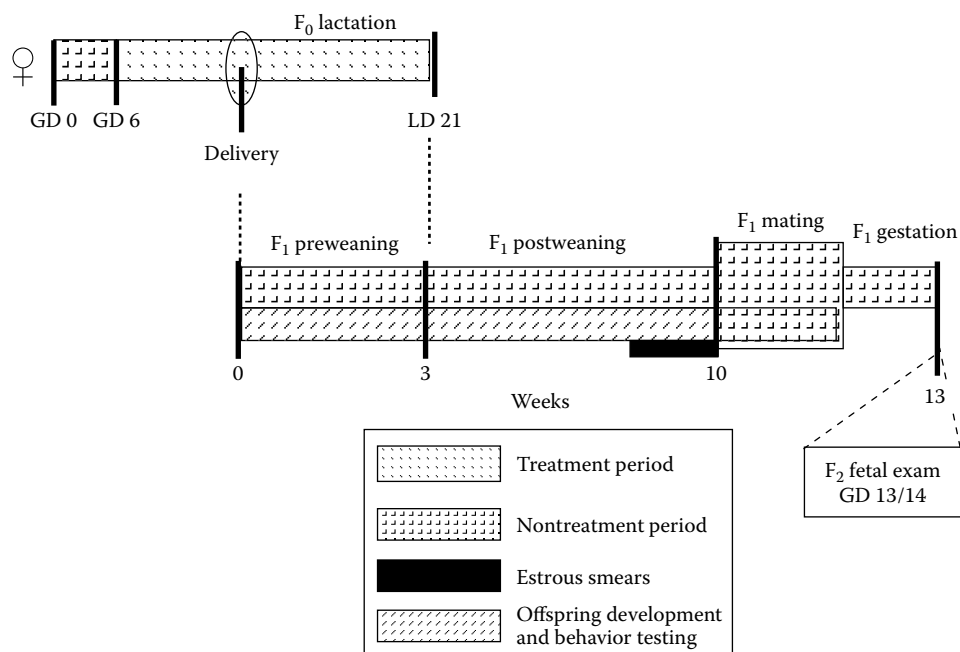


FIGURE 10.3 Prenatal and postnatal toxicity study schematics (ICH S5[R2]).

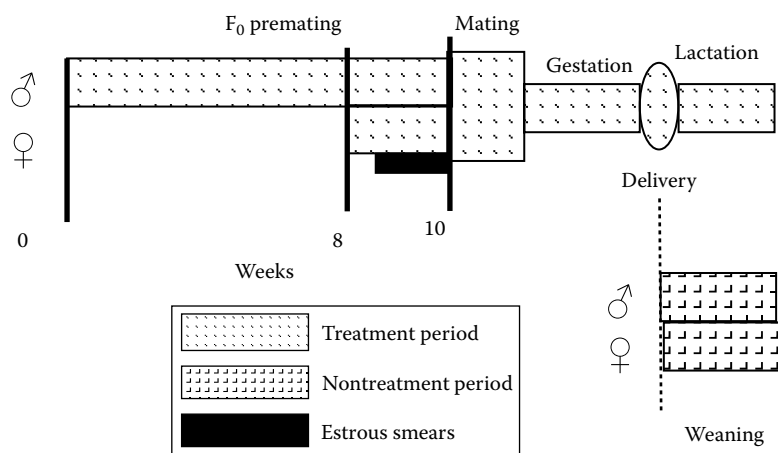


FIGURE 10.4 One-generation toxicity study schematics (OECD 415).

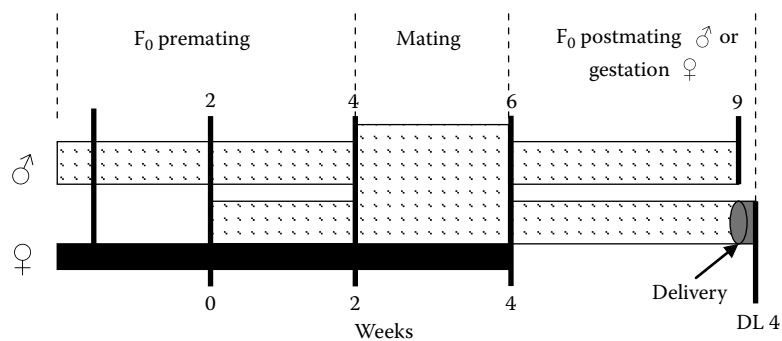


FIGURE 10.5 Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test schematic (OPPTS 870.3650 and OECD 422).

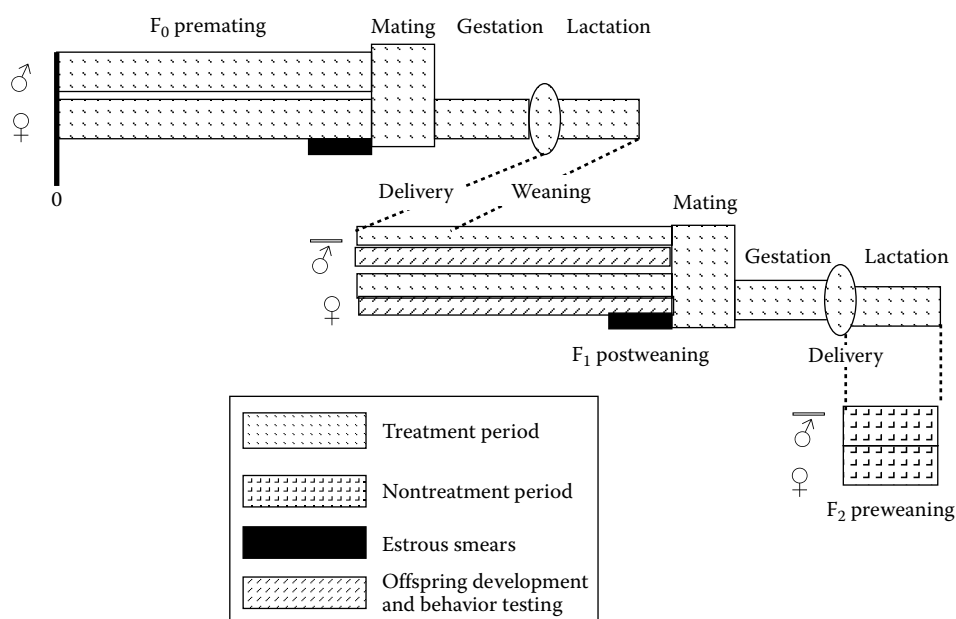
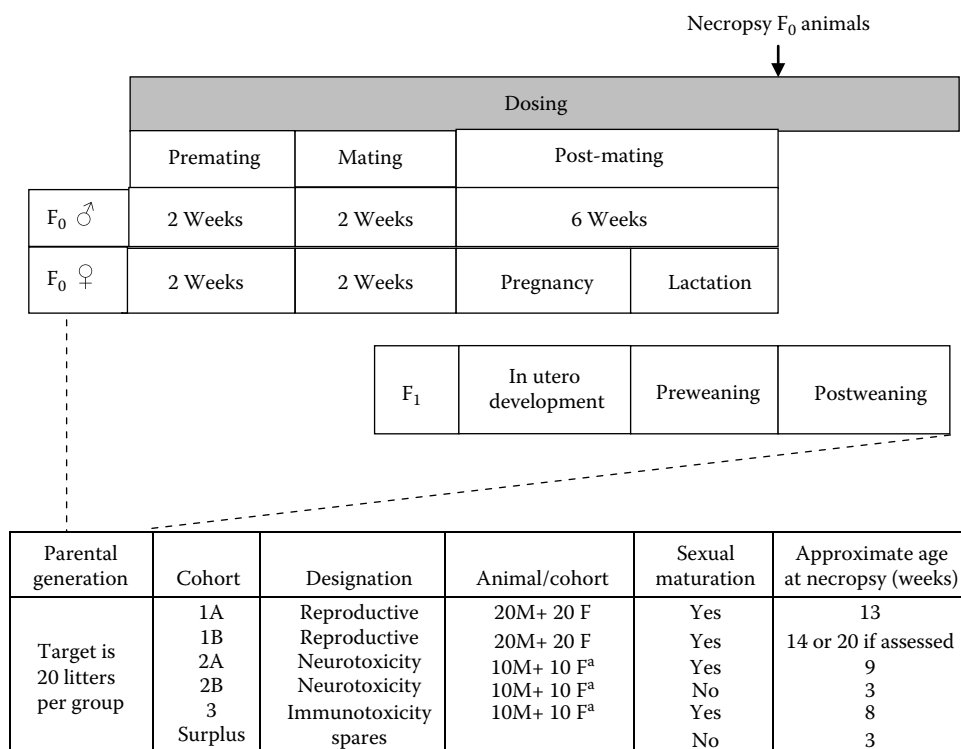


FIGURE 10.6 Two-generation reproduction study schematics (OPPTS 870.3800 and OECD 416).



^a one per litter and representative of 20 litters in total where possible

FIGURE 10.7 Extended one-generation toxicity study schematics (OECD).

MATERNAL AND DEVELOPMENTAL TOXICITY

Maternal toxicity may be evidenced by indicators such as body weight or weight gain, feed and water consumption, organ weights, gross or microscopic organ pathology, clinical signs of toxicity, and mortality (Table 10.2). Maternal toxicity may also be evidenced by indicators such as alterations in the estrous cycle or mating behavior, pre- and post-implantation losses, decreased corpora lutea or ovarian follicle counts, and altered weights and histopathology of the reproductive organs (Table 10.3).

Developmental toxicities are generally those that affect the F₁ or F₂ generations. The four manifestations of developmental toxicity as described by Wilson³⁶ are mortality, dysmorphogenesis (structural alterations), alterations to growth, and functional alterations (Table 10.4). Mortality due to developmental toxicity may occur at any time from conception to adulthood. Dysmorphogenic effects are seen as malformations or variations of the offspring. Alterations to growth are generally seen as growth retardation, although excessive growth or precocious maturation may also be seen. Functional alterations could include any persistent change in normal physiologic or biochemical function, but typically only reproductive function and developmental neurobehavioral effects are measured in these studies.

When the emphasis is on the appearance of birth defects rather than general developmental toxicity, be aware of the extremely short duration of the “target-window” in the animal surrogates. To produce birth defects rather than general

developmental toxicity may require a concentration of test agent that would kill the dam or destroy the pregnancy if delivered more than the 1 or 2 days included in the “target interval” (Figure 10.8).³⁷

The following information is provided as an overview of the complex process of normal development and also as an introduction to differences in developmental events in laboratory animal species and humans. Much of the following information is excerpted from York et al.,³⁷ and references cited within.

The development of a post-fertilization mammalian conceptus occurs within the uterine tubes and fundus of the uterus. After fertilization, there is a fusion of the genetic material of haploid egg and sperm to form a diploid zygote. Sperm activates the egg’s metabolism to start development. The development of the conceptus from the xygote to neonate is a complex process of extremely rapid cell proliferation and apoptosis (programmed cell death). In most laboratory species, the time from sperm penetration to first cleavage requires approximately 12 h. As the fertilized oocyte (zygote) travels down the uterine tube to the uterus, cleavage continues with growth to the morula stage. During this time, the zygote is surrounded by the zona pellucida, which previously prevented sperm penetration and now prevents premature implanting. In most mammals, the zona pellucida disappears at the blastocyst stage and the morula cavitates between 5 and 8 days of gestation. The preimplantation embryo has remarkable restorative growth potential, and it has been shown that a single cell from an eight-celled rabbit embryo can produce a normal offspring.

The preimplantation period was historically identified as a period during which toxic insult generally results in embryo death or absence of effect, due to the regenerative powers of the embryo. Currently, the preponderance of evidence shows all or nothing concept of “either death or recovery” does not always apply to preimplantation embryos (i.e., surviving embryos may demonstrate growth retardation, malformation, and/or functional impairment). Therefore, the entire embryonic and fetal stages of development are susceptible to toxic insult, and because many systems continue to develop after birth (e.g., lungs, immune system, reproductive organs), the postnatal period is also susceptible to toxic insult.

Cavitation is followed by attachment of the blastocyst to the uterine wall (nidation), and subsequent invasion of the uterine wall (implantation) by the syncytiotrophoblast, which erodes the endometrium. Placental circulation is subsequently established. Each blastocyst includes two different cell populations, the outer layer (trophoblast), which becomes the placenta and fetal membranes, and the inner cell mass (cluster of cells within the blastocyst), which becomes the embryo. Although sometimes not considered in the overall development of the embryo, the trophoblast serves an important function: the extraembryonic membranes protect the conceptus, while the placenta provides a means to supply nutrients and remove metabolic waste. There are multiple diverse mechanisms for placental transport of molecules, including both simple diffusion and carrier-mediated mechanisms (e.g., active transport, facilitated diffusion, receptor-mediated endocytosis). The placenta is clearly not a barrier, but rather as a means by which a substance in the maternal system will, at some rate and by some mechanism, be transported into the embryo. Remarkable differences exist across species with respect to the layers of embryonic and maternal tissues interposed between the respective circulations, and differences in the duration and functions of the yolk sac among species, which can affect the rate and access of a test material to a conceptus. Reproductive parameters and types of placenta and placentation for various species are shown in Tables 10.5 and 10.6.

The rapid growth of the conceptus continues through embryonic, fetal, and neonatal stages. The period of embryogenesis (organogenesis) is generally identified as the interval between implantation (and formation of the neural plate in the ectoderm) and closure of the hard palate (Table 10.7; Figure 10.9). The duration of these stages differs in various species and several classification schemes have been used to stage the embryonic development (Table 10.8). Key developmental events of various species are shown in Table 10.9.

Most organ systems are formed during this period, requiring cell proliferation, cell migration, cell–cell interactions, and morphogenetic tissue remodeling. Comparative development of organ systems (e.g., central nervous system [Table 10.10], eye and ear [Table 10.11], respiratory [Table 10.12], heart [Table 10.13], gastrointestinal tract [Table 10.14], plus the kidneys (Table 10.15), bone (Table 10.16), male and female reproductive systems (Tables 10.17 and 10.18), and immune

system (Table 10.19) of common laboratory species are described in greater detail in articles found in *Birth Defects Research* (Part B) Volume 68.

Each forming structure has a period of maximum susceptibility, with peak susceptibility to insult coinciding with the time key developmental events occur in these structures. The peak sensitivity may not only differ for a given tissue/organ but also with the administered dose. In addition, the same insult may affect the growth of concurrently developing systems. Thus, insult during the period of organogenesis is that most likely to result in gross structural malformations, although all end points of developmental toxicology have been shown affected, although the dose–response pattern for agents often differ, and the interrelationships of the responses often confound apparent dose-dependency.

The fetal period is characterized by tissue differentiation, growth, and physiological maturation. Almost all organs are present and grossly recognizable, and further development of these organs proceeds with the fetus attaining required functions before birth. These include fine structure morphogenesis (e.g., synaptogenesis, neural outgrowth, branching morphogenesis of the bronchial tree, and renal cortical tubules) and biochemical maturation (e.g., induction of tissue-specific enzymes and structural proteins). Insult during the period of fetogenesis is most likely to affect growth and functional maturation of the central nervous system, reproductive organs (including behavioral and motor deficits and reductions in fertility), the pulmonary system, and the immune system. Although gross structural changes can occur during the fetal period, such observations are generally secondary to deformations (changes in previously normal structures, such as clubbed or bent limbs), rather than malformations (abnormal growth).

TABLE 10.2
Signs of Overt Maternal Toxicity

1. Daily (or isolated) body weight changes and/or effects on food and/or water consumption during the dosing period^a
2. Changes in respiration, alertness, posture, spontaneous motor activity, color of mucous membranes, behavior (aggressive, depressed, lethargic, sedated), hair and coat appearance, color of urine, frequency of urination, and number and consistency of fecal pellets
3. Other signs such as nasal discharge, chromodacryorrhea, salivation, vaginal bleeding, tumor, convulsions, and coma
4. Death and necropsy findings

Source: Modified from Khera, K.S. et al., *Current Issues in Toxicology: Interpretation and Extrapolation of Reproductive Data to Establish Human Safety Standards*, Springer-Verlag, New York, 1989, Chapter 3.

^a Weight loss or failure to gain weight at any time during the dosing period may be followed by a rebound weight gain of sufficient magnitude to obfuscate an effect on maternal weight; therefore, maternal body weights should be determined daily.

TABLE 10.3
End Points of Developmental Toxicity in Female Rodents and Rabbits

Postconception evaluation
Maternal weight and body weight gain (daily during treatment; not in rabbits)
Clinical observations
Food consumption
Cesarean evaluations
Implantation number
Corpora lutea number (not in mice)
Gravid uterine weight
Litter size
Live fetuses and fetal weight (male, female, and total)
Deaths (embryonic, fetal)
Resorptions
Pup weight, crown-rump length
Incidence of malformations (external, visceral, skeletal, ossification sites)
Increased incidence of variations (external, visceral, skeletal)

Source: Modified from Parker, R.M., Testing for reproductive toxicity, in *Handbook of Reproductive Toxicology*, 3rd edn., Hood, R., Ed., CRC Press, Boca Raton, FL, 2011.

TABLE 10.4
Signs of Overt Embryofetal Toxicity

Mortality: Resorptions (early and late) and dead fetuses
Dysmorphogenesis (structural alterations): malformations or variations of the offspring
Alterations to growth: growth retardation, excessive growth or precocious maturation
Functional alterations: persistent change in normal physiologic or biochemical function, or reproductive function and developmental neurobehavioral effects.

Source: Modified from Wilson, J.G., *Environment and Birth Defects*, Academic Press, New York, 1973.

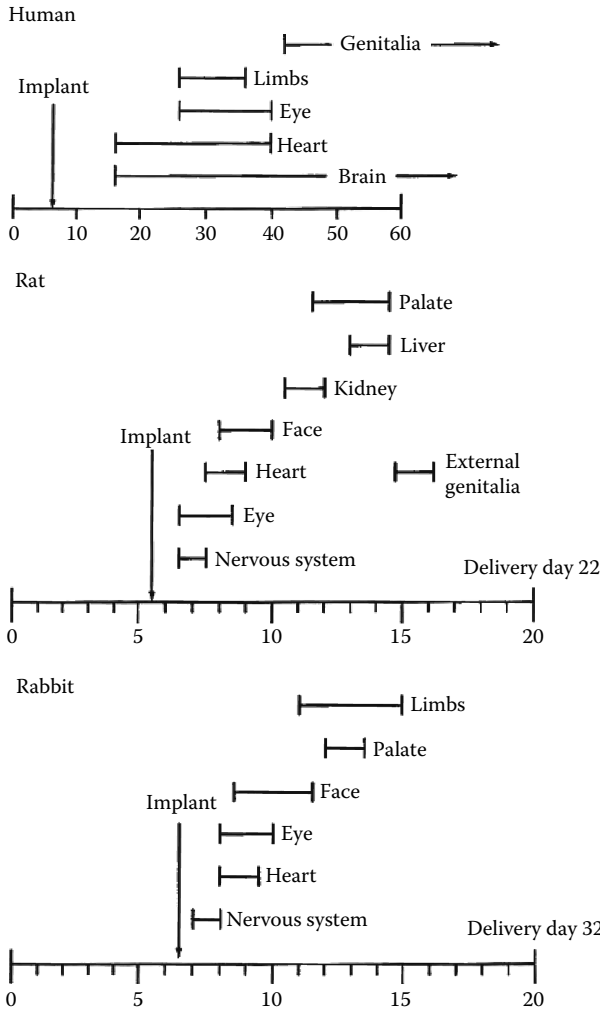


FIGURE 10.8 Critical periods of embryogenesis in the human, rat, and rabbit (days of gestation). (Modified from Ecobichon, D.J., Reproductive toxicology, in *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, Chapter 5, pp. 83–112, 1992.)

TABLE 10.5
Reproductive Parameters for Various Species

Species	Age at Puberty	Sexual Cycle		Ovulation		Copulation		Implantation (Days)	Gestation Period (Days)
		Type ^a	Duration (Days)	Time ^b	Type ^c	Time ^b	Length		
Mouse	5–6 weeks	PE	4	2–3 h	S	Onset of estrus		4–5	19 (19–21)
Rat	6–11 weeks	PE	4–6	8–11 h	S	1–4 h		5–6	21–22
Rabbit	6–7 months	PE	Indefinite	10 h	I	Anytime ^f	Sec	7–8	31 (30–35)
Hamster	5–8 weeks	PE	4	Early estrus	S	Estrus ^g		5+	16 (15–18)
Guinea Pig	8–10 weeks	PE	16–19	10 h	S	Estrus	Sec	6	67–68
Ferret	8–12 months	ME	Seasonal	30–36 h	I	Estrus	1–3 h	12–13	42
Cat	6–15 months	PE	Seasonal ^d	24–56 h	I	3rd day ^h	1–2 h	13–14	63 (52–69)
Dog	6–8 months	ME	9	1–3 days	S	Estrus	1–2 h	13–14	61 (53–71)
Monkey	3 years	PE	28	9–20 days	S	Anytime ⁱ		9	168 (146–180)
Man	12–16 years	PE	27–28	14 day (13–15)	S	Anytime	15–30 min	7.5	267 (ovulation)

Source: Modified from Spector, W.S., *Handbook of Biological Data*, WADC Technical Report 56-273, 1956.

^a PE, polyestrous; ME, monoestrous.

^b Time from start of estrous cycle.

^c I, induced ovulation; S, spontaneous ovulation.

^d March to August.

^e After mating.

^f Most receptive when in estrus.

^g 8–10 pm.

^h Of estrus, most receptive.

ⁱ Most receptive 2 days before ovulation.

TABLE 10.6
Type of Placenta and Placentation for Various Species

Species	Functional Yolk Sac ^a (Days/Somites)	Circulation ^b	Gross Shape	Type of Placentation ^c Relation to Endometrium		Implantation (Days)
				A	B	
Mouse	5–9/20	I	Discoid	Deciduate	Hemochorial	4–5
Rat	8–12/20	I	Discoid	Deciduate	Hemochorial	5–6
Rabbit	7–11/20	I	Discoid	Deciduate	Hemochorial	7–8
Hamster	6–9/20	I	Discoid	Deciduate	Hemochorial	5+
Guinea pig	8–19/20	I	Discoid	Deciduate	Hemochorial	6
Ferret	Hemophagous organ	?	Discoid	Deciduate	Endotheliochorial	12–13
Cat	Hemophagous organ	?	Zonary to Discoid	Deciduate	Endotheliochorial	13–14
Dog	Hemophagous organ	?	Zonary to Discoid	Deciduate	Endotheliochorial	13–14
Monkey (Rhesus)	12–22/5	III	Discoid	Deciduate	Hemochorial	9
Man	12–21/5	III	Discoid	Deciduate	Hemochorial	7.5

Sources: Modified from Spector, W.S., *Handbook of Biological Data*, WADC Technical Report 56-273, 1956; Beck, F. and Lloyd, J.B., Comparative placental transfer, in *Handbook of Teratology*, Wilson, J.G. and Fraser, F.D., Eds., Plenum Press, New York, 1977, Chapter 5, pp. 155–183; Dawes, G.S., *Foetal and Neonatal Physiology: A Comparative Study of the Changes at Birth*, Year Book Medical Publishers, Chicago, IL, 1968, Chapter 2.

Note: ?, unknown

^a Histotrophic nutrition; yolk sac main source of nutrition until last day/somite indicated.

^b Placental vascular channels.

^c Hemotrophic nutrition; A, Huxley's classification; B, Grosser's classification.

TABLE 10.7
Derivation of Various Organs from Primary Germ Layers of the Embryonic Inner Cell Mass

Endoderm	Mesoderm	Ectoderm (Trophoblast)
Liver	Vertebrae	Brain
Pancreas	Muscle	Spinal cord
Serous, mucous, and gastric glands	Dermis of skin, bone, and cartilage	Pituitary
Epithelium of digestive tube, respiratory system, and bladder	Connective tissue, blood, and blood vessels	Neural crest cells, retina, and lens
Thyroid	Urinary and reproductive apparatus	Adrenal medulla
Parathyroid	Epithelia of adrenal cortex, spleen, lymph nodes	Enamel of teeth
Pancreatic islets		Epithelium of oral, nasal, olfactory, genital, and anal cavities
Thymic corpuscles		Epithelium of skin and its derivatives—hair, nails, sweat, sebaceous, and mammary glands

Source: Modified from Hafez, E.S.E., *Reproduction in Farm Animals*, Lea & Febiger, Philadelphia, PA, 1968.

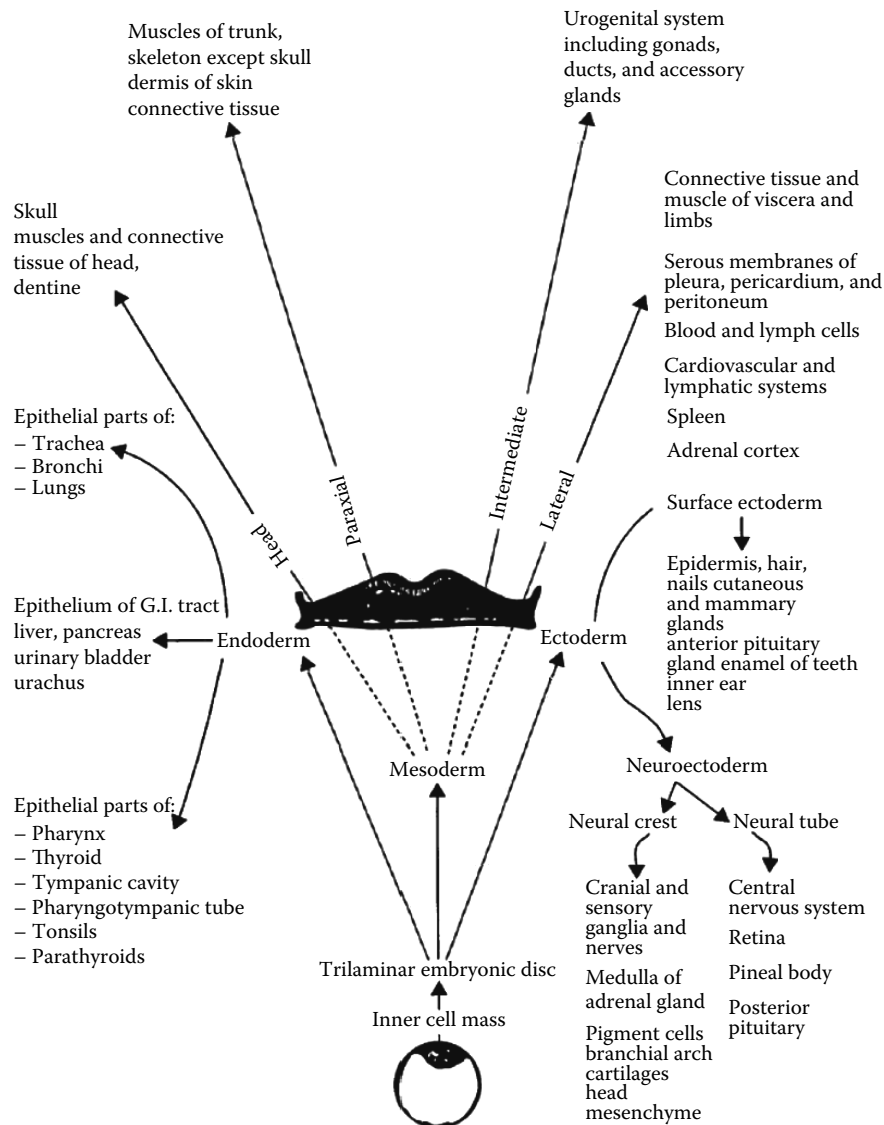


FIGURE 10.9 Derivation of various organs from primary germ layers of the embryonic cell mass. (Modified from Moore, K.L., *The Developing Human*, W.B. Saunders Co., Philadelphia, PA, p. 74, 1993.)

TABLE 10.8
Developmental Classification of Human Embryos

Developmental Stage	Witschi Standard Stages	Streeter Horizons	Carnegie Stages
Cleavage and blastula	1–7	I–III	1–3
Gastrula	8–11	IV–VII	4–6
Primitive streak	12	VIII	6–7
Neurula	13–17	IX–XII	7–12
Tailbud embryo	18–24	XII–XIII	12–13
Complete embryo	25	XIV	14
Metamorphosing embryo	26–33	XV–XXII	15–22
Fetus	34	XXIII	23
	34–36		Fetal period

Sources: Modified from Hoar, R.M. and Monie, I.W., Comparative development of specific organ systems, in *Developmental Toxicology*, Kimmel, C.A. and Buelke-Sam, J., Eds., Raven Press, New York, p. 13, 1981; O’Rahilly, R., *Development Stages in Human Embryos. Part A: Embryos of First Three Weeks (Stages 1–9)*, Publication 631, Carnegie Institute, Washington, DC, 1973; Streeter, G.L., *Contrib. Embryol.*, 30, 211, 1942; Streeter, G.L., *Contrib. Embryol.*, 31, 27, 1945; Streeter, G.L., *Contrib. Embryol.*, 32, 133, 1948; Witschi, E., *Development of Vertebrates*, W.B. Saunders, Philadelphia, PA, 1956.

TABLE 10.9
Times of Selected Key Developmental Events (in GDs)

Species	Implantation	Primitive Streak	10-Somite Stage	Lower Limb Buds	Hand (Forepaw) Rays	Palatal Folds Uniting	Gestation Period
Man	7.5	17	25	32	37	57	267
Macaque	9	17	23	28	35	46	167
Guinea pig	6.5	13	15	18.5	22	26	67
Rabbit	7.5	7.25	8.5	11	14.5	19.5	32
Rat	6	9	10.5	12	14	17	22
Mouse	5	8	1.5	10.3	12.3	15	19
Hamster	5	7	8	9.75	11	12	16

Source: Modified from Hoar, R.M. and Monie, I.W., Comparative development of specific organ systems, in *Developmental Toxicology*, Kimmel, C.A. and Buelke-Sam, J., Eds., Raven Press, New York, p. 13, 1981, which includes Refs. [48–50,52–69].

Note: GD, gestational day.

TABLE 10.10
Comparative Development of Nervous System (in GDs)

Species	Neural Plate	Neuropores Closed ^a	Three Brain Vesicles	Cerebral Hemispheres	Cerebellum	Olfactory Bulbs
Man	19	25–27	26	30	37	37
Macaque	20	25–27	25	29	36	38
Guinea pig	13.5	15.25–15.5	15.3	17	19	23
Rabbit	8	9.5–10.5	9.5	11	15	14
Rat	9.5	10.5–11	10.5	12	14	13.5
Mouse	7	9.0–9.5	8	10	12	11
Hamster	7.5	8.5–9.0	8.5	9	11	11

Sources: Modified from Hoar, R.M. and Monie, I.W., Comparative development of specific organ systems, in *Developmental Toxicology*, Kimmel, C.A. and Buelke-Sam, J., Eds., Raven Press, New York, p. 13, 1981 references as in Tables 10.7 and 10.8.

Note: GD, gestational day.

^a Anterior neuropore first; posterior neuropore second.

TABLE 10.11
Comparative Development of Eye and Ear (in GDs)

Species	Optic Vesicle Forming	Lens Separated	Optic Nerve Fibers Present	Optic Vesicle Forming	Cochlea Appearing	Optic Capsule Cartilaginous
Man	24	35	48	25	44	56
Macaque	23	32	39	25	37	42
Guinea pig	15.5	18	21.5	15.5	20.5	22
Rabbit	9	11.5	15	9	13	20
Rat	10.5	12.5	14	11.5	13.5	15
Mouse	9.5	11.5	13	8.5	12	14.5
Hamster	8	10	11	8.5	10	11

Source: Pei, Y.F. and Rhodin, J.A.G., *Anat. Rec.*, 168, 105, 1970, and as in Tables 10.7 and 10.8.

Note: GD, gestational day.

TABLE 10.12
Comparative Development of the Lungs (in GDs)

Species	Stage of Lung Development			
	Glandular	Canalicular	Saccular	Alveolar
Human	42–112	112–196	196–252	252–childhood
Rat	13–18	19–20	21–PND 7	PND 7–21
Mouse	14–16	16.5–17.4	17.4–PND 5	PND 5–
Rabbit	19–24	24–27	24–27	—
Sheep	–95	95–120	120–	—

PND, postnatal day.

Lung Growth in Humans and Rats
(Numbers Represent the Fold Change from Newborn to Adult)

Pulmonary Parameter	Human	Rat
Lung volume (mL)	23.4	23.5
Parenchyma airspace volume (mL)	30.2	26.9
Septal volume (mL)	13.5	13.6
Alveolar surface area (m ²)	21.4	20.5
Capillary surface area (m ²)	23.3	19.2

Source: Adapted from Zoetis, T. and Hurtt, M.E., *Birth Def. Res. (Part B)*, 68, 121, 2003 and references cited within.

Note: GD, gestational day.

TABLE 10.13
Comparative Development of Heart and Great Arteries (in GDs)

	Fusing Heart Tubes	S-Shaped Heart	Septation Occurring	Foramen Oval Present	Truncal Septation Complete	Aortic Arch Arteries Forming
Man	21	25	28	44	46	22–32
Macaque	22	25	28	34	36	22–30
Guinea pig	15	16	19.5	21	22	15.5–21.5
Rabbit	8.5	9.5	13	14	16.5	9–11
Rat	9.5	10	11.5	13	15.5	10–12
Mouse	7	8.5	10.5	12	14	8.5–11
Hamster	8	8.5	9.25	13	15	8–9.5

Sources: Modified from Hoar, R.M. and Monie, I.W., Comparative development of specific organ systems, in *Developmental Toxicology*, Kimmel, C.A. and Buelke-Sam, J., Eds., Raven Press, New York, p. 13, 1981; Sissman, N.J., *Am. J. Cardiol.*, 25, 141, 1970.

Note: GD, gestational day.

TABLE 10.14
Comparative Development of Gastrointestinal System/Time (in GDs)

Species	Intestinal Pocket		Membranes Perforate		Liver		Gall	Stomach	Umbilical Hernia	
	Foregut	Hindgut	Oral	Anal	Anlage	Epithelial Cords	Bladder	Appears	Begins	Reduced
Man	20.5	21.5	28.0	49	24.0	26.0	26.0	31–32	45.0	65.0
Macaque	20.5		27–28		24–26		28–29	28–29	33–34	47–48
Guinea pig	14.5	15.5			16.0	16.5	19.0	16.5	23.0	
Rabbit	8.5	9.0	10.0	10	9.5	10.5	11.5	10.5	12.5	20.0
Rat	9.5	11.0	10.0	15	11.0	11.5		11.5	12.5	18.0
Mouse	7.8	8.5	8.0	14	8.8	9.5	9.7	11.5	11.0	16.3
Hamster	7.8	8.0	8.5	13	8.5	9.0	8.7	8.5	9.3	13.0
Standard stages	13.0	15	15.0	31	16.0	16.0	17.0	18.0	27	34.0
Streeter's horizons	IX	XI	XI	XVIII	XI	XII	XII	XI–XII	XVII	XXIII

Source: Modified from Hoar, R.M. and Monie, I.W., Comparative development of specific organ systems, in *Developmental Toxicology*, Kimmel, C.A. and Buelke-Sam, J., Eds., Raven Press, New York, p. 13, 1981.

Note: GD, gestational day.

TABLE 10.15
Comparative Development of the Kidney

Species	Metanephros (GD)	Total Gestation Period (Days)	Timing of Nephrogenesis Completion
Man	35–37	267	Gestation week 35
Macaque	38–39	167	Birth
Guinea pig	23	67	Before birth
Rabbit	14	32	Postnatal week 2–3
Rat	12.5	22	Postnatal week 4–6
Mouse	11	19	Before birth
Dog	—	—	Postnatal week 2
Pig	—	—	Postnatal week 3
Sheep	—	—	Before birth

Source: Adapted from Zoetis, T. and Hurtt, M.E., *Birth Def. Res. (Part B)*, 68, 111, 2003.

Note: GD, gestational day.

TABLE 10.16

Age of Appearance and Fusion of Secondary Ossification Centers in the Humerus and Femur

	Age			
	Appearance		Fusion	
	Proximal Epiphysis	Distal Epiphysis	Proximal Epiphysis	Distal Epiphysis
Humerus				
Human	Gestation week 36–4 years	6 months–10 years	12–20 years	11–19 years
Monkey	Birth	Birth–1 month	4–6 years	1.75–4.5 years
Dog	1–2 weeks	2–9 weeks	10–12 months	6–8 months
Rabbit	1 day	1 day	32 weeks	32 weeks
Rat	8 days	8–30 days	52–181 weeks	31–158 days
Mouse	5–10 days	5–19 days	6–7 weeks	3 weeks
Femur				
Human	1–12 years	Gestation week 36–40	11–19 years	14–19 years
Monkey	Birth–6 months	Birth	2.25–6 years	3.25–5.75 years
Dog	1 week–4 months	2–4 weeks	6–13 months	8–11 months
Rabbit	1–5 days	1 day	16 weeks	32 weeks
Rat	21–30 days	8–14 days	78–156 weeks	15–162 weeks
Mouse	14–15 days	7–9 days	13–15 weeks	12–13 weeks

Source: Adapted from Zoetis, T. et al., *Birth Def. Res. (Part B)*, 68, 86, 2003.

TABLE 10.17

Comparative Development of the Male Reproductive System

	Human	Nonhuman Primate	Rat	Dog
Part 1				
Developmental stages				
Neonatal	To birth	To birth	Birth–7 days	Birth–4 days
Preterm				
Neonatal	Birth–1 month	0–2 weeks	7–14 days	4 days–3 weeks
Infantile	1 month–2 years	2–6 months	14–21 days	3–6 weeks
Children	2–12 years	6–36 months	21–45 days	6–20 weeks
Adolescent	12–16 years	36–48 months	45–90 days	20–48 weeks
Anogenital distance		Similar to rat	2.5 × 4 in males compared to females	
Preputial separation	Begins during late gestation; Complete from 9 months to 3 years of age. Androgens play key role		Sprague–Dawley PND 42–46; Androgens play key role.	
Puberty	12–14 years	2.5 years of age	Early puberty begins at PPS (<i>ca.</i> PND 43)	34–36 weeks as defined by the presence of ejaculated sperm
Prostate structure	Not sharply demarcated and appears as a single gland with several zones. (Lateral, dorsal, and medial lobes)		Discrete lobes (ventral lateral, dorsal, and paired anterior lobes, also known as coagulating glands). Dorsal and lateral lose distinct borders at adulthood. Lobes form between PND 1–7, tubular lumen between PND 7–14, secretory granules PND 14–21, and shows adult cytology PND 28–35.	The only well-developed accessory sex gland in the dog. Completely surrounds the urethra and divided into right and left lobes with middle lobe either poorly developed or absent. Prostate secretion starts at B4 months.

TABLE 10.17 (continued)
Comparative Development of the Male Reproductive System

	Human	Nonhuman Primate	Rat	Dog
Part 2				
Developmental stages				
Neonatal	To birth	To birth	Birth–7 days	Birth–4 days
Preterm				
Neonatal	Birth–1 month	0–2 weeks	7–14 days	4 days–3 weeks
Infantile	1 month–2 year	2 weeks–6 months	14–21 days	3–6 weeks
Children	2–12 years	6–36 months	21–45 days	6–20 weeks
Adolescent	12–16 years	36–48 months	45–90 days	20–48 weeks
Seminal vesicles (SV)	SV present by gestational month (GM) 6; adult form by GM 7. Growth of the SV continues slowly until puberty.		Basic pattern present at PND 10; lumen formation from PND 2–15; marked size increase PND 11–24; secretory granules PND 16; adult appearance/secretory properties PND 40–50.	No SV or bulbourethral glands.
Spermatozoa	Spermatogonia increase sixfold between birth and 10 years, then increase at puberty. Mean age at spermarche is 13.4 years	Spermatogonia more numerous by the end of the 1st year; spermatozoa in the testis appear at ca. 3 years; fertility at ca. 3.5–5 years	In seminiferous tubules PND 45; in vas deferens PND 58–59	In testis at 26–28 weeks of age; visible in the epididymis 26–28 weeks (beagles); first in ejaculate 32–34 weeks (fox terriers)
Leydig cells	Leydig cells begin producing testosterone (T) 7–8 weeks of gestation; pituitary gonadotrophin synthesis (human chorionic gonadotrophin) begins at 12 weeks of gestation.	Leydig cells are prominent in fetal life but decrease in number during the first year and dedifferentiate; at the end of 3 years, the Leydig cells redifferentiate.	Testosterone production begins late gestation; decreases prior to birth, PND 8–35 (infantile/juvenile period); testosterone–primary androgen by ca. PND 25.	First visible histologically GD 36–46; testicular LH receptors increase at 2 months (testicular max. 12–24 months); testicular T and DHT levels increase at 6 months, plateau 12–24 months.
Part 3				
Developmental stages				
Neonatal	To birth	To birth	Birth–7 days	Birth–4 days
Preterm				
Neonatal	Birth–1 month	0–2 weeks	7–14 days	4 days–3 weeks
Infantile	1 month–2 years	2 weeks–6 months	14–21 days	3–6 weeks
Children	2–12 years	6–36 months	21–45 days	6–20 weeks
Adolescent	12–16 years	36–48 months	45–90 days	20–48 weeks
Sertoli cells	Sertoli cell (SC) differentiation and production of anti-Mullerian hormone begins end of first trimester and is triggered by an unknown mechanism mediated by the Y chromosome.		Increase in number at GD 16, peak division GD 19, cease division PND 14–16. FSH receptors on SCs increase markedly before birth and peaks BPND 18 with a decline until adult levels PND 40–50	First visible GD 36–46 (beagles); divide up until 8 weeks postpartum; stable thereafter

(continued)

TABLE 10.17 (continued)
Comparative Development of the Male Reproductive System

	Human	Nonhuman Primate	Rat	Dog
Testis descent	Testis descent occurs prenatally	Testes descend at birth but soon after birth ascend into the inguinal canal (postnatal regression); testes descend at 3 years while increasing in size.	Testes attached to internal inguinal ring GD 20–21; testes into scrotum PND 15	Passage of testes through inguinal canal begins PND 3–4; descent complete at ca. PND 35–42
Epididymal ontogeny			Undifferentiated PND 0–15, differentiation PND 16–44, and expansion >PND 44	Postnatally: low columnar epithelium in all segments from birth to 20 weeks postpartum; Diameter of lumina increases slowly until week 20, after which there is a burst of growth, which levels off at approximately week 48 (caput), 36 (corpus), or 48 (cauda)

Source: Adapted from Marty, M.S. et al., *Birth Def. Res. (Part B)*, 68, 125, 2003 and references cited within.

TABLE 10.18
Comparative Development of the Female Reproductive System

	Human	Nonhuman Primate	Rat	Dog
Part 1				
Developmental stages				
Neonatal	To birth	To birth	Birth–7 days	Birth–4 days
Preterm				
Neonatal	Birth–1 month	0–2 weeks	7–14 days	4 days–3 weeks
Infantile	1 month–2 years	2 weeks –6 months	14–21 days	3–6 weeks
Children	2–12 years	6–36 months	21–45 days	6–20 weeks
Follicle maturation	Follicles were seen in 86% of prepubertal girls and in 99% of pubertal girls.	Not available	Ovarian follicles become subjected to strong gonadotropin control during the second week of postnatal life.	5–6 months: primary follicles show antrum formation.
Ovulation	In most young women, ovulation does not occur until six or more months after menarche, and a regular recurrence of ovulatory menstrual cycles does not appear to become established until several years later 12–24 months post menarche; Ovarian function last >30 years	The postmenarcheal phase of development in the rhesus monkey is the result of a high incidence of anovulatory and short luteal-phase cycles.	First ovulation ranges from about day 29 to day 38.	8–9 months to 12–14 months

TABLE 10.18 (continued)
Comparative Development of the Female Reproductive System

	Human	Nonhuman Primate	Rat	Dog
Reproductive tract maturation				
Uterine maturation	Uterine growth begins before puberty	Not available	Differentiation of the muscular and glandular epithelium: uterine glands first appear on PND 9 and increased until Day 15; Prepubertal: Small uterus, wet weight less than 100 mg with no intrauterine fluid; Postpubertal: Larger uterus, wet weight greater than 200 mg. Increase in uterine weight on Day 27.	Puberty
Part 2				
Developmental stages				
Neonatal	To birth	To birth	Birth–7 days	Birth–4 days
Preterm				
Neonatal	Birth–1 month	0–2 weeks	7–14 days	4 days–3 weeks
Infantile	1 month–2 years	2 weeks –6 months	14–21 days	3–6 weeks
Children	2–12 years	6–36 months	21–45 days	6–20 weeks
Reproductive tract maturation				
Anogenital distance	Not determined	Not determined	Anogenital distance is 3.5 mm for males and approximately one-half that for females; GD21: females, 1.29–1.41 mm; 3.15–4.44 mm for males; PND 0: females, 1.29–1.51 mm; males, 3.27–3.83 mm; Anogenital distance varies with body weight–normalization.	Not determined
Vaginal opening	Not determined	Not determined	CrI SD: 31.6–35.1 days; mean, 33.4 days; Occurs on the day after the first preovulatory surge of gonadotropins.	Not determined
Puberty	8–10 years: appearance of labial hair, initiation of breast enlargement; Menarche at 8–14 years.	2.5–3 years, nipple growth, increase in perineal swelling and coloration	Vaginal opening and first ovulation at ~5 weeks.	8–12 months; 5–13 months (mean, 9 months) in 8–15 kg beagles
Estrous cyclicity/menarche (first estrus/mensus)	10–16.5 years; mean, 13.4 years	2–3 years	36.4 days \pm 1.2 (SE).	8–12 months
Sexual maturity/fertility	11–16 years	2.6–3.5 years	50 \pm 10 days	8–12 months

(continued)

TABLE 10.18 (continued)
Comparative Development of the Female Reproductive System

	Human	Nonhuman Primate	Rat	Dog
Part 3—Endocrine Status				
Estrogen/estradiol	8–10 years, increased estradiol concentrations at initial stages of puberty	2.5–3 years, increased estradiol concentrations at initial stages of puberty	Range of free estradiol concentration during early neonatal period is similar to that during cycling adult	Not available
LH	Gonadotropes acquire the capacity to respond to the stimulatory action of estradiol on LH and FSH secretion, probably after menarche; GnRH neurosecretory system is active in neonatal period, then enters a dormant state; An increase in pulsatile release is essential for onset of puberty	Gonadotropes acquire the capacity to respond to the stimulatory action of estradiol on LH and FSH secretion after menarche; GnRH neurosecretory system is active in neonatal period, then enters a dormant state; An increase in pulsatile release is essential for onset of puberty	LH levels increase shortly after birth to a maximum on PND 12, then declines to about one-fifth by the end of the juvenile period. Prepubertal increase in LH 8–9 days before the expected day of first proestrus	Gonadotropes acquire the capacity to respond to the stimulatory action of estradiol on LH secretion by Day 20 and full capacity by Day 28; By 4 months, FSH concentrations are similar to those in adult anestrus
FSH	Gonadotropes acquire the capacity to respond to the stimulatory action of estradiol on LH and FSH secretion probably after menarche	Gonadotropes acquire the capacity to respond to the stimulatory action of estradiol on LH and FSH secretion after menarche	FSH levels increase shortly after birth to a maximum on Day 12, then declines to about one-fifth of the Day 12 values by the end of the juvenile period	By 4 months, FSH concentrations are similar to those in adult anestrus
Prolactin	No luteotropic effect in humans	No luteotropic effect in monkeys	Luteotropic in rats and mice; Anterior pituitary prolactin content and prolactin-containing cells increase with postnatal age, but prolactin levels remain low until the prepubertal period	Luteotropic role is questionable
Leptin	Adequate levels essential for onset of puberty and maintenance of cyclicity and reproductive function	In “monkeys,” adequate levels essential but not sufficient for onset of puberty; Adequate levels essential for onset of puberty and maintenance of cyclicity and reproductive function; Adequate levels essential for onset of puberty	Necessary for maintenance of estrous cyclicity; Adequate levels essential but not sufficient for onset of puberty; Adequate levels essential for onset of puberty and maintenance of cyclicity and reproductive function	Present but role in reproduction not defined.

Source: Adapted from Beckman, D.A. and Feuston, M., *Birth Def. Res. (Part B)*, 68, 137, 2003 and references cited within.

TABLE 10.19
Summary of Immune System Development across Multiple Species

Parameter	Human (GW, %)	Primate (GD, %)	Dog (GD, %)	Mouse (GD, %)
Gestation	40 weeks	155–165 days ^a	60–63 days ^b	20 days
Stem cells appear	5 (12.5)	—	—	8 (40)
Fetal liver hematopoiesis	7 (17.5)	—	—	10 (50)
Splenic primordia	10 (25)	—	28 (45)	13 (65)
Spleen demarcation ^c	26 (65)	80 (50)	45 (72)	—
Thymic primordia	6 (15)	35 (22)	28 (45)	10 (50)
Bone marrow hematopoiesis	12 (30)	—	45 (72)	18 (90)
Mitogen proliferation ^d	12 (30)	—	50 (81)	17 (85)

Source: Adapted from Holsapple, M.P. et al., *Birth Def. Res. (Part B)*, 68, 321, 2003 and references cited within.

Notes: GD, gestational day; GW, gestational week. Number in parentheses is the % of total length of gestation.

^a Length of gestation affected by strain of primate use 160 days for calculation.

^b Assume a gestational period of 62 days for later calculations.

^c Demarcation refers to the organization of spleen into distinct red and white pulp areas.

^d Proliferation in response to mitogens (PHA, Con A) by fetal thymocytes.

GLOSSARY OF TERMS RELATING TO DEVELOPMENTAL TOXICOLOGY

Modified from Middle Atlantic Reproduction and Teratology Association⁷⁸ and Terminology of Developmental Abnormalities in Common Laboratory Mammals (Versions 1 [1997]⁷⁹ and 2 [2009]⁸⁰).

Fetal and Newborn

General

Anasarca (edema): Generalized accumulation of serum in the intercellular space

Hydramnios: Excessive amniotic fluid

Monozygotic twins: Two fetuses derived from one zygote (fertilized ovum); one placenta

Polysomatos (polysomus): Doubling or tripling of the body of a fetus with each component having all or some of the body parts of a complete individual

1. Conjoined twins: Double fetuses ranging from two well-developed individuals (symmetrical conjoined twins) to those in which one incompletely developed fetus (unequal, parasitic twin) is attached to a more completely developed fetus (autosite)
2. Parasitic twins: Thoracopagus

Rudiment: Imperfectly or incompletely developed organ or body part having little or no function but which has functioned at an earlier stage of the same individual or in his/her ancestors

Subcutaneous hematoma: A circumscribed blood effusion in the subcutaneous tissue

Head

Cranium

Acephaly: Congenital absence of the head

Acrania: Partial or complete absence of the skull

Anencephaly: Congenital absence of the cranial vault with missing or small brain mass

Cephalocele: A protrusion of a part of the cranial contents, not necessarily neural tissue

Craniorachischisis: Exencephaly and holorrhachischisis (fissure of the spinal cord)

Cranioschisis: Abnormal fissure of the cranium; may be associated with meningocele or encephalocele

Dilatation of lateral ventricle: Enlargement of the lateral ventricle due to cerebrospinal fluid pressure; not so severe as to be called hydrocephaly

Domed head: Dome-shaped head; may or may not be associated with hydrocephaly

Encephalocele: Partial protrusion of brain through an abnormal cranial opening; not as severe as exencephaly

Exencephaly: Brain outside of skull as a result of large cranial defect

Hydrocephaly: Enlargement of the head caused by abnormal accumulation of cerebrospinal fluid in subarachnoid space (external hydrocephaly) or ventricular system (internal hydrocephaly)

Hydroencephalocele: Brain substance expanded into a watery sac protruding through a cleft in the cranium; a hernia through a cranial defect of brain substance and meninges, in which fluid occupies the space between the two

Meningocele: Hernial protrusion of the meninges through a defect in the skull

Meningoencephalocele: Hernial protrusion of brain and meninges

Microcephaly: Small head

Face

Eyes

Ablepharia: Absence or reduction of the eyelid(s)

Aniridia: Absence, complete or partial, of the iris

Anophthalmia: Absence of eye(s)
Aphakia: Failure of the lens to form
Cataract (opaque lens): Opacity of the crystalline lens
Coloboma: A cleft in the iris, ciliary body, choroid, or lids
Cyclopia: One central orbital fossa with none, one, or two globes
Exophthalmos: Protrusion of the eyeball ("pop" eye)
Folded retina: Detachment of the retina from the choroid
Macrophthalmia (buphthalmos): Enlarged eye(s)
Microphthalmia: Small eye(s)
Open eye(s): Split or unfused eyelid(s); may be due to
 ablepharia

Ear (Pinna)

Anotia: Absence of the external ear(s)
Ectopic pinna: Displaced external ear

1. Low set ear(s)—low placement of the external ear:

Microtia: Small external ear
Otocephaly: Absence of the lower jaw and ears united below
 the face
Synotia: Persistence of the external ears beneath the
 mandible

Nose

Absent or micro concha nasalis (enlarged nasal, turbinate(s))
 (see "Enlarged nasal turbinate(s)")
Arrhinia: Absence of nose
Enlarged nasal turbinate(s): Enlargement of the nasal
 passage(s) due to absent or micro concha nasalis
Naris (nostril) atresia: Absence or closure of nares
Nasal agenesis: Absence of the nasal cavity and external nose
Nasal atresia: See "Naris (nostril) atresia"
Rhinocephaly: A developmental anomaly characterized by
 the presence of a proboscis-like nose above the eyes,
 partially or completely fused into one
Septal agenesis: Absence of nasal septum
Single nostril: Single external naris

Mouth/Jaws

Aglossia: Absence of the tongue
Agnathia: Absence of lower jaw (mandible)
Astomia: Absence of oral orifice
Bifid tongue: Cleft tongue
Cheilognathopalatoschisis: A cleft in the hard and soft pal-
 ate, upper jaw and lip
Cleft face: Incomplete fusion of embryonic processes that
 normally unite in the formation of the face or one
 of its parts
Cleft lip: Incomplete fusion of the lip
Cleft palate: Incomplete fusion of the palatine shelves
High-arched palate: A higher than normal palatal arch
Macroglossia: Enlarged tongue, usually protruding
Median facial cleft: Affects lips and maxilla or palate
Micrognathia: Shortened lower jaw (mandible); tongue may
 protrude

Microstomia: Small mouth opening
Protruding tongue: Tongue protruding from the mouth
 opening; could be the result of macroglossia or
 micrognathia
Schistoglossia: Cleft tongue

Trunk

General

Achondroplasia: A hereditary defect in the formation of
 epiphysal cartilage, resulting in a form of dwarfism
 with short limbs, normal trunk, small face, normal
 vault, etc.
Exomphalos: Congenital herniation of abdominal viscera
 into umbilical cord
Gastroschisis: Fissure of abdominal wall (median line) not
 involving the umbilicus, usually accompanied by
 protrusion of the small and part of the large intes-
 tine, not covered by membranous sac
Omphalocele: Midline defect in the abdominal wall at the
 umbilicus, through which the intestines and often
 other viscera (stomach, spleen, and portions of the
 liver) protrude. These are always covered by a mem-
 branous sac. As a rule, the umbilical cord emerges
 from the top of the sac

1. Umbilical eventration—see "Umbilical hernia"

Situs inversus viscerum (totalis or partialis): Total or
 partial transposition of viscera (due to incomplete
 rotation) to the other side of the body; heart most
 commonly affected (dextrocardia)
Thoracogastroschisis: Midline fissure in the thorax and
 abdomen
Thoracoschisis: Fissure of the chest wall
Umbilical hernia: Protrusion of viscera at the navel, covered
 by skin

Esophagus/Trachea

Ectopic esophagus: Displacement of the esophagus (descrip-
 tion of position should be included)
Esophageal atresia: Discontinuity of the esophageal
 lumen usually associated with tracheoesophageal
 fistula
Esophageal stenosis: Constriction or narrowing of the
 esophageal lumen
Tracheal stenosis: Constriction or narrowing of the tracheal
 lumen
Tracheoesophageal fistula: Communication between
 esophageal and tracheal lumen

Heart/Major Vessels

**Abnormal origin of right common carotid and right
 subclavian artery:** Both arteries directly off
 the arch, associated with absence of innominate
 artery
Acardia: Absence of the heart

Atrioventricular (A–V) septal defect: A defect that results in communication between an atrium and ventricle

Atrioventricular ostium (orifice) enlarged: Enlargement of an atrioventricular orifice

Atrioventricular valve enlarged: See previous

Cardiomegaly: Hypertrophy (enlargement) of the heart

Cor biloculare: Two-chambered heart

Cor triloculare: Three-chambered heart

Dextrocardia: Location of the heart in the right side of the thorax; a developmental disorder that is associated with total or partial situs inversus (transposition of the great vessels and other thoracoabdominal organs) or occurs as an isolated anomaly

1. Secondary dextrocardia—displacement of the heart to the right side of the thorax as a result of disease of the pleura, diaphragm, or lungs

Ectocardia: Displacement of the heart inside or outside the thorax

1. Ectopic cordis—displacement of the heart outside of thorax caused by a failure of the midline to close (sternal cleft)

Enlarged pericardial sac: Enlargement of the sac (cavity) that envelops the heart

Exocardia: Abnormal position of the heart

Inferior vena cava defect:

1. Dilated—lumen of the inferior vena cava enlarged or expanded
2. Displaced—inferior vena cava out of normal position

Levocardia: Displacement of the heart in the extreme left hemithorax

Innominate artery agenesis: Absence of the innominate artery (which normally arises from the arch of the aorta); associated with separate origin of the right common carotid artery and the right subclavian artery from the aortic arch

Interatrial septal defect (Foramen ovale apertum): A defect that results in communication between the atria. This defect is produced by abnormal development of the septum primum and septum secundum

Interventricular septal defect: A defect of the septum between the ventricles of the heart; usually located at its membranous (superior) portion

Monocardium: Possessing a heart with only one atrium and one ventricle

Patent ductus arteriosus (ductus botalli): An open channel of communication between the main pulmonary artery and the aorta; may occur as an isolated abnormality or in combination with other heart defects

Right-sided descending aorta: An aorta descending on the right side instead of the left side

Situs inversus: See next

1. Cardiovascular situs inversus—Mirror-image transposition of the heart and vessels to the other side of the body

Tetralogy of Fallot: An abnormality of the heart that includes pulmonary stenosis, ventricular septal defect, dextraposition of the aorta overriding the ventricular septum and receiving blood from both ventricles, right ventricular hypertrophy

Transposition of great vessels: The aorta originates from the right ventricle, whereas the pulmonary artery arises from the left ventricle; often associated with interventricular septal defect or a patent ductus arteriosus

Truncus communis: A common aortic and pulmonary truncus; usually associated with other heart/vessels defects

Lung

Agenesis of the lung (lobe): Complete absence of a lobe of the lung

Aplasia of the lung: The trachea shows rudimentary bronchi, but pulmonary and vascular structures are absent

Atelectasis: Incomplete expansion of the lungs (or portion of the lung) at birth; collapse of pulmonary alveoli during postnatal life

Hypoplasia of the lung: Bronchial tree poorly developed, and pulmonary tissue shows an abnormal histologic picture (total or partial); incomplete development, smaller

Unilobular lung: In the rat fetus, a condition in which the right lung consists of one lobe instead of four separate lobes

Abdomen/Abdominal Viscera

Diaphragm

Diaphragmatic eventration: Elevation of the dome of the diaphragm

Diaphragmatic hernia/diaphragmatocele: Protrusion of viscera, usually liver or intestine, through a defect in the diaphragm

Stomach

Agastria: Absence of the stomach

Dextrogastric: Having the stomach on the right side of the body

Gastromegaly: Abnormal enlargement of the stomach

Liver

Hepatic lobe agenesis: Absence of a lobe of the liver

Hepatomegaly: Abnormal enlargement of the liver

Multilobular liver: Greater than normal number of liver lobes

Gallbladder

Agenesis of gallbladder: Absence of the gallbladder (Note: Rats do not have a gallbladder)

Multiple gallbladder: More than one gallbladder (can be bilobed)

Intestines

Agenesis of intestine: Absence of the intestine or section thereof

Kidneys

Absent or reduced renal papilla: Absent or smaller than usual apex of a Malpighian pyramid in the kidney

Agenesis of the kidney: Absence of the kidney(s)

Dilatation of renal pelvis: Abnormal distention of the pelvis due to urine retention or delayed development of renal papilla

Double kidney: Duplication of the renal pelvis and ureter in one kidney (in some cases, the separation of the duplicated organ is incomplete [fused supernumerary kidney])

Ectopic kidneys: A congenital anomaly in which the kidney(s) is located in an abnormal position

1. Pelvic kidney—location of the kidney in the pelvic region

Enlarged kidney(s): Larger than normal size

Fused kidneys: Fusion of both kidneys

1. Cake (lump) kidney—extreme fusion in which both kidneys unite to form one kidney
2. Disk (donut) kidney—fusion of both the upper and lower poles
3. Horseshoe kidney—fusion of the lower poles (common) or the upper poles (rare)
4. Sigmoid kidney—fusion of the lower pole with the upper pole of the contralateral kidney

Hydronephrosis: Dilatation of the renal pelvis usually combined with destruction of renal parenchyma and often with dilatation of the ureters (bilateral, unilateral) (Note: This is a pathology term and should have histological confirmation)

Renal hypoplasia: Incomplete development of the kidney

Supernumerary kidney: A “kidney” in addition to the two usually present

Ureters

Aplasia of the ureter: Failure of the ureter(s) to develop

Convoluted ureters: Twisting (coiling) of the ureter(s)

Adrenals

Adrenal agenesis: Absence of the adrenal(s)

Adrenal hypoplasia: Underdeveloped adrenal(s)

Enlarged adrenals (adrenal hyperplasia, hypertrophy): Larger than normal-sized adrenal(s)

Spleen

Accessory spleen: An additional spleen

Asplenia: Absence of the spleen

Enlarged spleen: Larger than normal size

*Urinary Bladder/Gonads/Anus**Ureters*

Acystia: Absence of the urinary bladder

Gonads

Anorchism: Congenital absence of one or both testes

Cryptorchidism (undescended testes, ectopic testes): Failure of the testes to descend into the scrotum (can be unilateral)

Hermaphroditism: Presence of both male and female sex organs in one individual

Hypospadias: Urethra opening on the underside of the penis or on the perineum (males), or into the vagina (females)

Pseudohermaphroditism: Possession of the sex organs of one sex (ovary or testis) but accompanied by some secondary sexual characteristics and external genitalia more or less similar to those of the opposite sex

Anus

Anal atresia: Congenital absence of the anus

Aproctia: Imperforation or absence of anus

Imperforate anus: Persistence of the anal membrane, so that the anus is closed (often associated with atresia of the lower portion of the rectum)

*Skeletal System**General*

Agenesis: Absence

Asymmetrical: Lack of correspondence in size, shape, or position of a pair of bones or bone parts on both sides of the median plane

Bent: Slightly curved

Bipartite ossification: Two ossification sites visible

Displaced (malpositioned): Out of normal position

Duplicated: A doubling

Fused bones: Joining of bones which should not be joined

Incomplete ossification (delayed, retarded): Extent of ossification is less than what would be expected for that developmental age; not necessarily associated with reduced fetal or pup weight

Irregular ossification: Uneven calcification of cartilage matrix; abnormal sequence in appearance of ossification sites

Misshapen bones: Bone that differs in shape from normal

Missing: Absent

Rotated: Turning or tipping of a structure with the center of the point maintained

Shortened: Reduced in length

Thickened: Increased in extent from one surface to the opposite one

Unossified: Absence of ossification; existing structure present as a cartilaginous or membranous element

Skull See “General” (“Developmental Toxicology” section)

Angulated hyoid: Abnormally shaped hyoid, in which greater cornua forms sharper than normal angles with the hyoid body

Vertebrae See “General” (“Developmental Toxicology” section)

Extra vertebrae: In excess of the normal number of vertebrae

Hemivertebra: Presence of only one half of a vertebral body

Lordosis: Anterior concavity in the curvature of the cervical and lumbar spine as viewed from the side

Lumbosacral shift: Condition when the lowest lumbar arch is aligned with the first sacral arch

Rachischisis: Absence of vertebral arches in a limited area (partial rachischisis) or entirely (rachischisis totalis)

Scoliosis: Appreciable lateral curvature of the vertebral column

Spina bifida: Defect in closure of bony spinal canal:

1. Spinal bifida cystica—spina bifida associated with spinal cord and (Aperta) meninges’ protrusion
2. Spina bifida occulta—opening covered by skin; no protrusion of the spinal cord or meninges

Centra See “General” (“Developmental Toxicology” section)

Dumbbell: Two ossification centers connected by a narrow isthmus

Hemicentra: Ossification of only one site within a centrum

Sternebrae See “General” (“Developmental Toxicology” section)

Asymmetrical: Ossification sites are uneven, but not in a set pattern

Checkerboard: Ossification centers are bipartite and staggered giving checkerboard pattern

Hemisternebrae: Incomplete or accessory development of one side of a sternebral body

Ribs See “General” (“Developmental Toxicology” section)

Bulbous: Having a bulge or balloon-like enlargement somewhere along its length

Displaced: Out of normal position

Rudimentary: Imperfectly developed rib-like structure

Short: Less than one-half the size of a normal rib

Thickened: Having a wide appearance

Wavy: Having curves; an undulating pattern

Extremities See “General” (“Developmental Toxicology” section)

Abasophalangia: Absence of the proximal phalanx

Abrachius: Without arms, forelimbs

Adactyly: Absence of digits

Amelia: See “Ectromelia”

Amesophalangy: Absence of medial phalanx

Ankylosis: Inflexible, stiff joint

Aphalangia: Absence of a finger or a toe; corresponding metacarpals not affected

Apodia: Absence of one or both feet

Arthrogryposis: Persistent flexure or contracture of a joint; flexed paw (bent at wrist) is the most common form of arthrogryposis

1. Dorsiflexed (hyperextension)—flexed dorsally

2. Plantar-flexed (hyperflexion)—flexed ventrally

Atelephalangy: Absence of a distal phalanx

Bowing of hind limbs: A bending outward of the limb(s)

Brachydactyly: Shortened digits

Club foot: Foot twisted out of shape or position, pes contortus, talipes

Dysmelia: Absence of a portion of one or several limbs

Ectrodactyly: Absence of all or of only a part of digit (partial ectrodactyly)

Ectromelia: Aplasia or hypoplasia of one or more bones of one or more limbs (this term includes amelia, hemimelia, and phocomelia)

Hemimelia: Absence of all or part of the distal half of a limb; could be: radial hemimelia, ulnar hemimelia, fibular hemimelia, or tibial hemimelia

Hyperflexed limbs: Excessive flexion of a limb

Macrobrachia: Abnormal size or length of the arm

Macroactyly: Excessive size of one or more digits

Oligodactyly: Fewer than normal number of digits

Peromelia: Congenital absence or deformity of the terminal part of a limb or limbs

Phocomelia: Absence of proximal portion of limb(s) with the paws being attached to the trunk of the body by a single small irregularly shaped bone

Polydactyly: Extra digits

Symphodia (syrenomelus, sympus): Fusion of the lower extremities

Synarthrosis: A form of articulation, almost immobile, in which the bony elements are united by fibrous tissue

Syndactyly: Partially or entirely fused digits

Synmetacarpals/synmetatarsals: Fused metacarpals or metatarsals

Tail

Acaudia (anury): Agenesis of the tail

Brachyury (short tail): Tail that is reduced in length

Coiled tail: Tail with a spiral curvature

Curled tail: Tail with a “curved” type of bend

Filamentous: Thread-like

Fleshy tab: Filamentous extension from the tip of an otherwise normal tail

Kinked tail: Tail with a distinct bend

Rudimentary tail: Tail that exists only as a fleshy element

Parental*Maternal**General*

Abortifacient: An agent that causes abortion

Abortion: The premature expulsion from the uterus of the products of conception; of the embryo or of a nonviable fetus

Estrus: Phase of the sexual cycle of female mammals characterized by willingness to mate; estrous, adjective (e.g., estrous cycle)

Fecundity: Ability to produce offspring rapidly and in large numbers

Fertility: Capacity to conceive or induce conception

Infertility: Absence of the ability to conceive or to induce conception

Parity: Condition of a female with respect to the number of pregnancy(ies) that resulted in viable born offspring

Mammary Glands

Lactation: (a) The secretion of milk; (b) the period of the secretion of milk

Supernumerary mammae: Accessory breast tissue and/or nipples (teats); mammae accessoriae

Vagina

Vaginal plug: A mass of coagulated semen which forms in the vagina of animals after coitus; also called copulation plug or bouchon vaginal

Uterus

Ametria: Congenital absence of the uterus

Feticide: The destruction of the fetus in the uterus

Hydrometra (uterine dropsy): Excess fluid (clear, colorless) in the uterus

Pyometra: Pus within the uterus

Uterus: The hollow muscular organ in female animals in which the developing embryo and fetus are nourished

Ovaries

Corpus luteum (corpora lutea, pl.): The yellow endocrine body formed in the ovary at the site of the ruptured Graafian follicle

Follicle: Small excretory or secretory sac or gland

1. Atretic—a graafian follicle that has involuted
2. Graafian—a small spherical vesicular sac embedded in the cortex of the ovary, which contains an egg cell or ovum; each follicle contains a liquid, liquor ovarii, and produces hormone folliculin or estrin
3. Ovarian—the egg and its encasing cells at any stage of its development
4. Primordial—an ovarian follicle consisting of an undeveloped egg enclosed by a single layer of cells:

Luteal: Pertaining to or having the properties of the corpus luteum, its cells, or its hormone

Luteinic: (a) Pertaining to or having the properties of the corpus luteum; (b) pertaining to luteinization

Luteinization: The process taking place in the follicular cells of graafian follicles that have matured and discharged their egg; the cells become hypertrophied and assume a yellow color, the follicles becoming corpora lutea

Pregnancy

Ectopic pregnancy: Implantation (and possible development) of a blastocyst outside of the uterine cavity

Multigravida: A female pregnant for the second (or more) time

Multipara: A female that has had two or more pregnancies, which resulted in birth of viable offspring

Nulliparous: A female that never has born viable offspring

Pseudopregnancy: (a) False pregnancy: condition occurring in animals in which anatomical and physiological changes occur similar to those of pregnancy; (b) the premenstrual stage of the endometrium, so called because it resembles the endometrium just before implantation of the blastocyst

Unigravida (primigravida): A female pregnant for the first time

Unipara (primipara): A female who has borne only one offspring

Uniparental: Pertaining to one of the parents only

Uniparous (primiparous): Producing only one ovum or offspring at one time

Parturition

Delivery: Expulsion or extraction of the fetus and placenta at birth

Parturition: The act or process of giving birth

Dystocia: Abnormal labor

Labor: Function of female organism by which the product of conception is expelled from the uterus through the vagina to the outside world

1. Complicated—labor in which convulsions, hemorrhage, or some other untoward event occurs
2. Delayed—labor which occurs later than the expected date
3. Premature (early)—labor which occurs before the normal end of gestation

Perinatal: Occurring shortly before, during, or shortly after birth

Postparturition (postpartum): After birth, or after delivery

Products of Conception

Blastocyst: The mammalian conceptus in the postmorula stage

Conceptus: The sum of derivatives of a fertilized ovum at any stage of development from fertilization until birth

Implantation (nidation): Attachment of the blastocyst to the epithelial lining of the uterus, including its penetration through the uterine epithelium, and its embedding in the endometrium

Morula: The solid mass of blastomeres (embryonic cells) formed by cleavage of a fertilized ovum

Placental Membranes

Amniochorial: Pertaining to the amnion and chorion

Amnion: The innermost of the fetal membranes; a thin, transparent sac which holds the fetus suspended in the amniotic fluid

Chorion: The outermost of the fetal membranes, consisting of an outer trophoblastic epithelium lined internally by extraembryonic mesoderm; its villous portion, vascularized by allantoic blood vessels, forms the placenta

Oligohydramnios (oligoamnios): Reduction in the amount of amniotic fluid

Polyhydramnios: Excessive amount of amniotic fluid

Yolk sac: An extraembryonic membrane composed of endoderm and splanchnic mesoderm; it is the organ in which the first red blood cells are formed; in rodents, it is the primary absorptive surface (gut) prior to formation of the placenta

Ova

Gamete: A male (spermatozoon) or female (ovum) reproductive cell

Implantation (nidation): Attachment of the blastocyst to the epithelial lining of the uterus, including its penetration through the uterine epithelium, and its embedding in the endometrium

Embryo

Conceptus: The whole product of fertilization until birth; the embryo or fetus and the extraembryonic membranes

Embryogenesis: The growth and development of the embryo

Embryogenic: (a) Pertaining to the development of the embryo; (b) producing an embryo

Embryo: The early or developing stage of any organism, especially the developing product of fertilization of an egg after the long axis appears and until all major structures are presented

Embryonal: Pertaining to embryo

Embryonic: Pertaining to the embryo as defined earlier

Embryopathology: The science of abnormal embryos or of defective development

Extraembryonic: Not occurring as a part of the embryo proper; applied specifically to the fetal membranes

Presomite: Embryos before the appearance of somites

Somite: One of the paired masses of mesoderm arranged segmentally alongside the neural tube of the embryo, forming the vertebral column and segmental musculature

Teratoma: A tumor containing a disorderly arrangement of tissues and organs, as a result of faulty embryonic differentiation and organization produced through abnormal inductive influences

Fetus

Conjoined twins: Double fetuses, ranging from two well-developed individuals joined by a superficial

connection of varying extent, to those in which only a small part of the body is duplicated

Fetal: Pertaining to a fetus

Fetoplacental: Pertaining to the fetus and placenta

Fetus: The unborn offspring in the postembryonic period

Neonatal: Pertaining to a newborn offspring (in the human, the first 4 weeks of life)

Neonate: Newly born; a newborn offspring

Polysomia: A doubling or tripling of the body of a fetus

Terata: A malformed fetus with deficient, redundant, misplaced, or misshapen parts (plural: terata)

Paternal

General

Androgen: A class of steroid hormones produced in the gonads and adrenal cortex that regulate masculine sexual characteristics

Cycle of the seminiferous epithelium: The complete series of successive cellular associations occurring in the seminiferous epithelium

Daily spermatozoal production: The total number of sperm produced per day by the two testes

Duration of spermatogenesis: The interval between the time a stem spermatogonium becomes committed to produce spermatozoa and its subsequent release from the germinal epithelium into the lumen (about 72 days in the human and fewer in most animals)

Efficiency of spermatozoal production: The number of sperm produced per day per gram of testicular parenchyma

Ejaculate: The total seminal sample obtained during ejaculation

Ejaculation: The expulsion of semen through the urethra

Fecundate (fertilize): To impregnate

Meiosis: Cell division occurring in maturation of the sex cells (gametes) by means of which each daughter nucleus receives half the number of chromosomes characteristic of the somatic cells of the species

Nonmotile spermatozoon: A sperm that does not move a discernible distance during visual observation

Percentage of motile sperm: The percentage of sperm that are motile, regardless of pattern (progressive, circular, or backward)

Percentage of progressively motile sperm: The percentage of sperm that moves forward (excluding circularly motile and backward motile sperm)

Semen: A mixture of sperm and fluids from the excurrent ducts and accessory sex glands

Seminiferous epithelium: The normal cellular components within the seminiferous tubule consisting of Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids

Sertoli cells: Cells in the testicular tubules providing support, protection, and nutrition for the spermatids

Sperm morphology: The characteristic features (shape, form) comprising sperm

Spermatogenetic cellular stage: One of a series of characteristic cellular groupings of different types of germ cells found in a specific area of a seminiferous tubule

Spermatocytogenesis: The first stage of spermatogenesis in which spermatogonia develop into spermatocytes and then into spermatids

Spermatogenesis: The process of formation of spermatozoa, including spermatocytogenesis and spermiogenesis

Spermiation: The second stage of spermatogenesis in which the spermatids transform into spermatozoa

Spermiogenesis: The second stage of spermatogenesis in which the spermatids transform into spermatozoa

Testosterone: The hormone produced by the interstitial cells of the testes, which functions in the induction and maintenance of male secondary sex characteristics

Total spermatozoa: The total number of spermatozoa in an ejaculate

Modifying Terminology

Development

Agenesis: Absence of an organ or part of an organ

Anomaly (anomalous): Any deviation (malformation or variation) from the norm

Aplasia: Lack of development of an organ, frequently used to designate complete suppression or failure of development of a structure from the embryonic primordium

Bifurcate: Forked

Bifurcation: (a) Division into two branches; (b) site where a single structure divides into two

Bilobate: Having two lobes

Defect: An imperfection

1. Acquired—an imperfection gained secondarily after birth, not hereditary or innate
2. Congenital—an imperfection present at birth, due to abnormal embryonic development

Deflection: A turning, or state of being turned, aside

Deformity: Distortion of any part or general disfigurement of the body

Development: Gradual growth or expansion, especially from a lower to a higher stage of complexity

1. Arrested—cessation of the developmental process at some stage before its normal completion
2. Postnatal—that which occurs after birth
3. Prenatal—that which occurs before birth
4. Regulative—the development of an embryo; the determination of the various organs and parts being gradually attained through the action of inductors

Deviation: Variation from the regular standard or course

Dislocation: The displacement of any part from the original position

Displacement: Removal from the normal position or place; ectopia

Diverticulum: A pouch or pocket leading off a main cavity or tube

Dysgenesis: Defective development; malformation

Dysplasia: (a) Abnormal development of tissue; (b) alteration in size, shape, or organization of adult cells

Ectopic: Out of the normal place

Evagination: An outpouching of a layer or part

Fissure: Any cleft or groove, normal or abnormal

Imperforate: Not open; abnormally closed

Macro: (prefix) Meaning large

Malformation: Defective or abnormal formation; deformity; a permanent structural deviation, which generally is incompatible with, or severely detrimental to, normal survival or development

Malposition: Abnormal or anomalous position

Micro: (prefix) Meaning small

Morphogenesis: The various processes occurring during development by which the shape and the structure of a particular organ or part of the body are established

Morphology: The science of the form and structure of an organism, or any of its parts, without regard to function

Pendulous: Hanging loosely; swinging freely

Variation: A minor divergence beyond the usual range of structural constitution

Consistency

Maceration: The softening of a solid by soaking; in obstetrics, the degenerative changes with discoloration and softening of tissues, and eventual disintegration of a conceptus retained in the uterus after its death

Mummification: Conversion into a state resembling that of a mummy, such as occurs in dry gangrene, or the shriveling and drying up of a dead fetus

Pachynsis: Abnormal thickening

Color

Albinic: Unpigmented, having no color

Albinism: Congenital absence of pigment in the skin, hair, and eye; may be total or partial

Cyanosis: Bluish discoloration, applied especially to skin and mucous membranes

Icteroid (icteritious): Having a yellow hue; seemingly jaundiced

Common Laboratory Terms

General

Aberration: A minor structural change; it may be a retardation (a provisional delay in morphogenesis), a variation (external appearance controlled by genetic and extragenetic factors), or a deviation (resulting from altered differentiation)

Abortion: The termination of pregnancy before the conceptus is capable of ex utero survival

Anomaly or abnormality: A morphologic or functional deviation from normal limits; it can be a malformation or a variation

Average pup (fetus) weight: Total weight of living pups (fetuses) in litter/number of living pups (fetuses) in litter

Birth: Delivery at term

Cannibalism: The eating of one's own kind, frequently observed in the postpartum period of rabbits or rats

Conceptus: Term used when the stage of prenatal development at the time of initial insult is not known; also referred to as embryo–fetus

Day “0” of lactation: Usually referred to as “postpartum day 0” day of birth

Day “0” of pregnancy: Day on which positive evidence of mating has been ascertained

Dead fetus: A nonliving fetus in utero

Developmental toxicity: Any adverse effect on development (morphologic, physiologic, or functional), initiated prenatally and appearing during the lifetime of the progeny

Embryo–fetus: Used when the stage of prenatal development at the time of conception is not known; also referred to as conceptus

Embryotoxicity: Signifies embryonic loss during the early preimplantation or postimplantation stages of pregnancy

Fetotoxicity: Any prenatally initiated toxic manifestation observed in a fetus (death, body weight reduction, delayed ossification, or functional defect), which may or may not be related to toxic effects in the mother and which, although initiated in utero, appears during prenatal or postnatal development; any of these effects resulting from the direct action of a test agent on the embryo or fetus (as defined in Dorland's Medical Dictionary) and occurring at doses far below those toxic for the mother should be regarded as suggestive of true, or selective, embryo- or fetotoxicity

Maternal toxicity: Transitory or permanent pathologic state of health or alteration in maternal physiology and/or behavior with the potential to cause adverse effects in the offspring during embryofetal or postnatal development

Positive evidence of mating: Sperm or semen plug in vagina

Postpartum: After delivery

Pregnant: An animal with uterine evidence of implantation or an animal which delivers a conceptus

Premature birth: Birth before expected time but capable of surviving ex utero

Reproductive toxicity: Deals with toxic effects on any aspect of reproduction of the offspring proceeding from the development of gametes and their fusion

Resorption: A conceptus which, having implanted in the uterus, subsequently died and is being or has been resorbed

Retarded development: An organ, fetus, or newborn which has not developed concomitantly with its chronological age

Retarded growth: Growth of fetus or neonate which is not concomitant with chronological age; small for age

Runt: Normally developed fetus or newborn that is significantly smaller than the rest of the litter

Stillbirth: Birth of dead fetus

Teratogen: An intrinsic or extrinsic factor or an alteration in maternal homeostasis that induces, during prenatal development, a permanent structural or functional abnormality in the fetus, which is detected in a prenatal or postnatal examination; if malformations are induced at apparently nonmaternotoxic doses, the agent is a true, or selective, teratogen

Term: The expected date of birth

Threshold level: The highest level of a chemical or test substance, obtained by the best possible estimates from experimental data that is judged insufficient to produce an adverse effect on prenatal and postnatal development in humans or animals

Weaning date: Day on which animal is separated from its mother

REPRODUCTIVE TOXICOLOGY

INTRODUCTION

Reproductive toxicology is “the study of the occurrence, causes, manifestations, and sequelae of adverse effects of exogenous agents on reproduction.”⁸¹ Reproductive “hazards” may encompass adverse health effects in the prospective mother, the father, and/or, of course, the developing conceptus. The most striking features of reproduction are the myriad of rapidly multiplying cells (e.g., ova, spermatozoa, the organs and tissues of the conceptus, placenta), and the susceptibility of such cells to a variety of agents at concentrations that may be significantly different than would be anticipated to elicit toxicity in other cell systems. This is a challenging field of toxicology, the spectrum of events caused by an *environment* (drugs, substances of abuse, industrial chemicals, pesticides, diet, lifestyles, etc.) resulting in effects as covert as alterations in normal levels of hormones with changes in performance (loss of libido, impotence, sterility, etc.) or more overt evidence of toxicity in the form of spontaneous abortion, embryonic or fetal death, reduced perinatal survival, or teratogenicity (structural and/or functional anomalies) (Figure 10.10).

One must appreciate the concept of *toxic windows* during gamete production/function and in embryonic development, the narrow periods during which particular cell systems are susceptible to chemical-induced damage. In reproductive toxicology, it is insufficient to determine that the target site of the agent is the testis, the ovary, the conceptus, etc., but to examine the variety of direct and indirect events occurring during the reproductive/development cycle, each of these is being characterized by multiple components (Table 10.20).

1. Early—evidence of implantation without recognizable embryo/fetus
2. Late—dead embryo or fetus with external degenerative changes

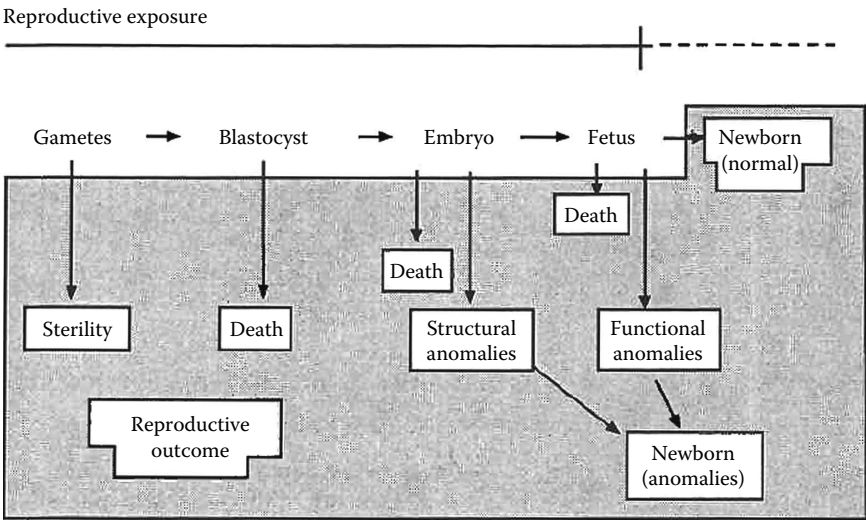


FIGURE 10.10 Physiological events during reproduction when exposure to physical or chemical agents or various forms of energy may induce adverse effects in reproductive outcomes.

TABLE 10.20
Reproduction—Development Cycle

Fecundity	Embryo formation
Libido	Differentiation
Gametogenesis	Organogenesis
Gamete	Fetal maturation
Transport	Parturition
Function	Neonatal
Mating	Viability
Fertilization	Development
Zygote transport	Lactation
Implantation	Nutrition
Placental	Postnatal maturation
Formation	Sexual maturation
Function	Gametogenesis

Source: Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992. With permission.

END POINTS OF REPRODUCTIVE TOXICITY

The end points of reproductive toxicity in test species can be divided into three categories: couple-mediated, male-specific, and female-specific.⁸² These topics will be briefly restated here.

Couple-Mediated End Points

Couple-mediated end points are reproductive end points where both parents may have a contributory role. Couple-mediated end points from reproduction studies may include (1) mating behaviors (mating rate, time to mating), (2) pregnancy rate, (3) preimplantation and postimplantation loss, (4) number of implantation sites, (5) gestation length,

(6) litter size (total and live), (7) number of live and dead offspring, (8) sex ratio, (9) birth weight, (10) external and internal malformations and variations, (11) postnatal weight, (12) postnatal structural and functional development, (13) offspring survival, and (14) offspring reproduction. Most regulatory reproduction and fertility guidelines specify cohabitation of treated male rats with treated female rats, complicating the resolution of gender-specific influences.

Male-Specific End Points and Paternally Mediated Effects

Data on the potential male reproductive toxicity of a test substance may be collected from many guideline studies,

including acute, subchronic, chronic, reproduction, and fertility studies. See Table 10.21 for the male-specific end points that are determined in these studies. Male-only treatment with a variety of agents has been shown to produce adverse effects in offspring, including pre- and postimplantation loss, growth and behavioral deficits, and structural malformations.^{83–85} Many of the chemicals reported to cause paternally mediated effects display genotoxic activity and may cause transmissible genetic alterations. However, other mechanisms of induction of male-mediated effects are possible,⁸⁶ including nongenetic (e.g., presence of drug in seminal fluid) or epigenetic (e.g., an effect on imprinted gene expression from the paternal alleles, resulting in alteration of gene function). Additional studies will be needed to clarify the mechanisms of paternal exposure associated with adverse effects on offspring. If a test substance is identified as causing a paternally mediated adverse effect on the offspring of the test species, the effect should be considered an adverse reproductive effect.

TABLE 10.21
Male-Specific End Points in Fertility Studies

Body weight
Mating behavior (not observed)
Libido
Mounting
Erection
Ejaculation
Reproductive organ weights
Histopathology
Testes
Epididymides
Seminal vesicles
Prostate
Pituitary
Sperm parameters
Quality
Count
Viability
Morphology
Motility
Transport
Maturation
Storage in the epididymis
Production of seminal fluid
Production and secretion of hormones in the pituitary–hypothalamus–gonadal axis.

Source: Adapted from Hood, R.D. and Parker, R.M., Reproductive and developmental toxicology, in *Preclinical Development Handbook*, Gad, S., Ed., John Wiley Press, New York, 2008.

Female-Specific End Points and Maternally Mediated Effects

The reproductive life cycle of the female may be divided into several phases (embryo/fetal, prepubertal, cycling adult, pregnant, lactating, and reproductively senescent). Detailed descriptions of these phases are beyond the scope of this chapter but are readily available.^{87,88} Detailed discussion of maternally mediated effects on development is beyond the scope of this chapter.⁸⁹ Studies should be conducted to detect adverse effects occurring during any of these phases. Regulatory developmental and reproductive studies primarily detect adverse effects on the female's ability to become pregnant, on pregnancy outcome, and on offspring survival and development. Adverse alterations in the nonpregnant female reproductive system have been reported at treatment levels below those that result in reduced fertility or produce adverse effects on pregnancy or pregnancy outcomes.^{90–92} See Table 10.22 for the female-specific end points of reproductive toxicity.

Unlike that of the male, the status of the adult female reproductive system fluctuates. In nonpregnant rodents, the ovarian and uterine structures (and other reproductive organs) change throughout the estrous cycle. Although not cyclic, other changes normally occur during pregnancy, lactation, and return to cyclicity after lactation. These normal fluctuations may affect or confound the evaluation of female reproductive end points. It is important to be aware of the reproductive status of the female at necropsy, including estrous cycle stage. This facilitates interpretation of effects with such end points as uterine weight and the histopathology of the ovary, uterus, and vagina.⁸⁶

TABLE 10.22
Female-Specific End Points in Fertility Studies

Body weight
Estrous cycle
Onset
Length
Patterns
Mating behaviors
Reproductive organ weights
Histopathology
Ovary
Uterus
Vagina
Ovulation
Number of corpora lutea
Tubal transport
Implantation
Pre- and post-implantation losses

Source: Adapted from Hood, R.D. and Parker, R.M., Reproductive and developmental toxicology, in *Preclinical Development Handbook*, Gad, S., Ed., John Wiley Press, New York, 2008.

SPECIES DIFFERENCES

Reproductive toxicology requires detailed knowledge of species differences in reproductive biology and development. No surrogate animal species, used routinely in reproductive toxicity studies, mimics exactly the human in reproductive physiology. Consider the dramatic differences in uterine structure between multiparous animals and those bearing only one conceptus. Clearly, certain species lend themselves to the study of agent-induced actions on certain facets of reproduction. Characteristically, the first problem facing the investigator in reproductive studies is the vast interspecies variability in biological end points that must be considered when choosing a suitable animal model before any study. For reproductive toxicity studies, at least one species, preferably rats with evaluation of between 16 and 20 litters per group for rodents provide sufficient numbers to allow meaningful interpretation of the data. Advantages and disadvantages of species (strains) should be considered in relation to the substance to be tested, the selected study design, and in the subsequent interpretation of the results. Rats, and to lesser extent mice, are good, general-purpose models. Mice have a fast metabolic rate, stress sensitivity, malformation clusters, limited size for toxicokinetic blood sampling, and small fetuses. The rabbit has been somewhat underused as a “nonrodent” species for reproduction studies other than embryotoxicity testing. The rabbit has attributes that would make it a useful model for fertility studies, especially male fertility. For both rabbits and dogs (which are often used as a second species for repeat dose toxicity studies), semen samples can easily be obtained (electro-ejaculation) for longitudinal semen analysis. For most other species, disadvantages often outweigh the advantages.

The male and female cynomolgus monkeys' reproductive physiology and endocrinology have been well-characterized and closely resemble that of humans making them well-established models for reproduction.^{94–96} The female cynomolgus monkey has clinically relevant parameters available for the assessment of female reproductive toxicity: oogenesis (histology), menstrual cyclicity, endocrine status (luteinizing hormone [LH], follicle stimulating hormone [FSH], CG, prolactin, estrogens, progesterone, androgens, inhibins), and fertility. The male cynomolgus monkey also has well-established and clinically relevant

parameters available for the assessment of male reproductive toxicity: spermatogenesis (histology, daily sperm production, and spermatogenic stages), sperm maturation, semen parameters, testis size/biopsy, endocrine status (LH, FSH, prolactin, estrogens, progesterone, androgens, and inhibins), prostate status, and fertility. The development of the cynomolgus monkey has also been well-established making it a model for developmental toxicology studies.⁹⁷ The available parameters for the assessment of developmental toxicity in the cynomolgus monkey include during pregnancy (pregnancy monitoring via ultrasound, hematology, clinical chemistry, body weight, and maternal blood hormone analysis); at cesarean section (amniotic fluid analysis, maternal/fetal blood analysis, fetal organ weights, fetal examination [external, visceral and skeletal], maternal/fetal immunoglobulins and antibodies, and fetal histology and immunocytochemistry); and during the postnatal period (infant growth and clinical signs, maternal/infant blood analysis, maternal/infant immunoglobulins and antibodies, milk analysis, behavioral and functional tests, reflex ontogeny and sexual maturation in the offspring). However, the use of monkeys for reproductive toxicology testing is not without several challenges. Sexual maturity for the female cynomolgus monkey is usually attained by the age of 3.5 years and at a weight of at least 3.0 kg. Additional measurements such as hormone concentration (progesterone and 17 β estradiol) and grading of the concentration/density of blood on the vaginal swab are considered unnecessary for confirmation of sexual maturity. Menstrual cycles and gestation and lactation periods are long (approximately 28 days, 165 days, and 10–11 months, respectively), resulting in studies of long duration. The menstrual cycle of cynomolgus monkeys has been divided into four stages: follicular (days 1.5–8.5); late follicular/ovulatory (ca. days 8.5–15.5); luteal (days 15.5–22.5); and premenstrual/menstrual (ca. days 22.5–1.5). Vogel⁹⁸ described criteria of maturity for male cynomolgus monkeys: (1) bodyweight > 4.5 kg, (2) age: 4–5 years; (3) testicular volume >10 mL (cross section \approx 2.2 cm in diameter), (4) serum testosterone > 15–20 nmol/L, and (5) proof of sperm in the ejaculate.

For the most commonly used test species, various parameters are listed in Table 10.23 for the breeding characteristics of the female of the species, with comparable data for the human.

TABLE 10.23

Breeding Characteristics of Female Laboratory Mammals Compared with the Human^a

Parameters	Monkey ^b	Dog	Cat	Rabbit	Mouse	Guinea Pig	Hamster	Rat	Human
Age at puberty (days)	36 months	6–8 months	6–15 months	5.5–8.5	35	55–70	35–56	37–67	12–15 years
Breeding season	All year	Spring–Fall	Feb–July	All year	All year	All year	All year	All year	All year
Breeding life (years)	10–15	5–10	4	1–3	1	3	1	1	35
Breeding age (months)	54	9	10	6–7	2	3	2	2–3	180
Estrous cycle (days)	28 ^c	22	15–28	15–16	4–5	16–19	4	4–5	27–28
Duration of estrus (days)	1–6 ^d	7–13	4–19	30	1	1	1	1	2–8
Gestation period (days)	164	63	63	31	20	67	16	21	267
Litter size (number)	1	3–6	1–6	1–13	1–12	1–5	1–12	6–9	1
Birth weight (g)	500–700	1100–2200	125	100	1.5	75–100	2.0	5.6	
Opening of eyes (days)	At birth	8–12	8–12	10	11	At birth	15	11	At birth
Weaning age (weeks)	16–24	6	6–9	18	3	2	3–4	3–4	
Weight at weaning (g)	4400	5800	3000	1000	11–12	250	35	10–12	

^a Data obtained from various sources including Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 2, Table 1; Spector, S., *Handbook of Biological Data*, W.B. Saunders Company, Philadelphia, PA, 1956, various tables; Altman, P.L. and Dittmer, D.S., *Biology Data Book*, 2nd edn., Vol. I, Federation of American Societies for Experimental Biology, 1972, various tables.

^b Monkey = *Macaca mulatta* or *fascicularis*.

^c Menstrual cycle.

^d Duration of menstruation.

GAMETOGENESIS**Male Gametogenesis**

Adult testicular function is largely influenced by the two gonadotrophic hormones, FSH and LH, both released from the anterior pituitary in response to the actions of the hypothalamic gonadotropin releasing hormone (GnRH). Each of these glycoproteins has specific tissue targets as is shown in Table 10.24. Additional hormonal influence comes from testosterone and estradiol originating from the Leydig cells and inhibin from the Sertoli cells, each with their own specific target site, usually in a negative feedback capacity.

Numerous articles,^{99,100} chapters^{101–106} and books^{107–109} have been written concerning the testis. The testis is covered by the tunica albuginea, a tough fibrous capsule. The rat testis contains very little intertubular connective tissue, in contrast to the human testis, which is partitioned by connective tissue septa. However, the rat testis is similar to the human testis in having two major compartments: the interstitial compartment and the seminiferous tubule compartment. The interstitial compartment contains the connective tissue, interstitial (Leydig) cells and stem cells, macrophages, nerves, blood vessels, and lymphatic vessels. Unlike human testicular capillaries, rat testicular capillaries are not fenestrated. The appearance of Leydig cells in the developing testes coincides with the production of testosterone. Pulses of LH from the pituitary cause the Leydig cells to release testosterone. Macrophages are commonly

observed in the interstitium and may account for up to 25% of the interstitial cells.

A limiting membrane or boundary tissue formed by lymphatic endothelial cells, myoid cells, and acellular components (basal lamina) bounds the seminiferous tubule compartment. The myoid cells are contractile and provide the major motive force for the movement of sperm and fluid through the seminiferous tubule. The basal lamina and the myoid cells provide the structural base on which the Sertoli cells and the basal compartment cells of the seminiferous epithelium rest. Sertoli cells extend around the periphery of the tubule through to the lumen, with invaginations surrounding germ cells at various stages of differentiation. Specialized junctions between the Sertoli cells form the blood–tubule barrier (also known as the blood–testis barrier) in the rat at approximately 18 days of age. After that, the Sertoli cells do not divide again. Sertoli cells maintain metabolic support for the seminiferous epithelium, maintain hormonal control of spermatogenesis and testicular function, and have a phagocytic function. Developing spermatozoa depend entirely on the Sertoli cells for nutrition, support, and position in the epithelium. The seminiferous tubules are highly convoluted loops that have their two ends connected to short segments of straight tubules (tubuli recti). Although highly convoluted, the tubules run parallel to the long axis of the testis. The tubuli recti connect to the rete testis that is in turn connected to the cephalic portion of the epididymis by 10–20 long and tortuous efferent ducts (ductuli efferentes).

Spermatogenesis begins during fetal life, stem cells being transformed to spermatogonia after birth, although these latter cells lie dormant until puberty when proliferative activity resumes (Table 10.25). Spermatogenesis can be divided into two separate stages: (1) *spermatocytogenesis*, during which the diploid Type A spermatogonia replicate by mitosis, each cell undergoing five mitotic divisions to form a host of Type B spermatogonia to be converted subsequently into primary spermatocytes and into haploid spermatids; (2) *spermiogenesis*, involving the differentiation of the spermatids into spermatozoa, the metamorphosis from a rounded cell into the characteristically shaped, mature spermatozoon having an elongated, condensed nucleus in the head, an acrosome, a reduction in cytoplasm, and a flagellum.^{42,43,110} When spermiogenesis is completed, the spermatozoon is released into the lumen of the seminiferous tubule. Further maturation in the epididymis results in fertility and motility being conferred on the spermatozoon and the generation of suitable fluid vehicle. This fluid is made up of secretions contributed by accessory glands (prostate), the seminiferous vesicles, and the epididymis. Agents may interfere with any of these functions or act on the epididymal tissue and/or the spermatozoon during this maturation period of 12 (rat) to 21 (human) days residence time.

Considerable interspecies variability exists in the developmental/maturation stages of spermatogenesis in the testis in transit times through the various sections of the epididymis and in the production rates of sperm (Table 10.26). All these factors must be considered in choosing an experimental model and the selection of time periods for treatment.

The most sensitive end point for the determination of testicular toxicity is histopathology. Testicular histology and sperm parameters are linked,¹¹¹ and alterations in testicular structure are usually accompanied by alterations in testicular function. However, some functional changes may occur in the absence of detectable structural changes in the testis. Alterations in the testicular structure and function are also linked to alterations in epididymal structure and function, but changes in epididymal function may occur in the absence of testicular or epididymal structural changes.

Depending on the degree of male immaturity, the testes are characterized by tubules that contain little to no germ cells or only partially populated with germ cells.⁸² The first wave of spermatogenesis is inefficient and often results in a large number of degenerate and exfoliated cells. During this initial spermatogenic wave, not all seminiferous tubules develop at the same rate; therefore, some tubules may appear fully functional and mature while other tubules that are not as advanced may appear "atrophic." These rapidly dividing, developing, and maturing cells are highly susceptible to chemical insult at many stages (Figure 10.11). Testicular toxicants can produce changes that are virtually indistinguishable from the immature seminiferous tubule. Therefore, examination

of the testes of an immature or peripubertal animal can provide a confusing impression, which may be improperly interpreted (either as a false-positive or a false-negative). In many dogs that are <6 months old or in monkeys that are <3 years old, spermatogenesis has not begun. When such immature animals are used, severe testicular toxicants may not be detected because there are no spermatogenic cells to be affected.

Histopathological evaluations of the testis can determine whether sloughed cells are present in the tubule lumen, whether the germinal epithelium is degenerating or severely depleted, and whether multinucleated giant cells are present. The rete testis should be examined for dilation caused by obstruction or disturbances in fluid dynamics and for the presence of proliferative lesions and rete testis tumors. Both spontaneous and chemically induced lesions in the rete testis often appear as germ cell degeneration and depletion. More subtle lesions, such as missing germ cell types or retained spermatids, can reduce the number of sperm being released into the tubule lumen. Such effects may not be detected when less than adequate methods of tissue preparation have been used. Also, knowledge of the details of testicular morphology and spermatogenesis assists in the identification of lesions that may accompany lower treatment levels or result from short-term exposure.

Microscopic evaluation of the testis should be a qualitative evaluation carried out with an awareness of the spermatogenic cycle. Quantitative procedures are inappropriate for screening studies. Several methodologies for qualitative or quantitative assessment of testicular tissue, including the use of "spermatogenic cycle staging," can assist in the identification of testicular lesions.¹¹² To provide high-resolution cellular morphologic detail required for spermatogenic cycle staging, semi-thin (2 μ m) sections will have to be produced from testes embedded in glycol methacrylate resin. A detailed description of spermatogenesis and spermiogenesis required for staging is beyond the scope of this chapter; however, detailed morphological descriptions with light and electron microscopic photographs of these processes are available in an excellent book by Russell et al.¹¹¹

Rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility, particularly as evaluated in reproductive studies allowing multiple matings.^{113,114} In some strains of rats and mice, sperm production can be drastically reduced (by up to 90% or more) without affecting fertility.^{115–117} Human male sperm production appears to be much closer to the infertility threshold; therefore, less severe sperm count reductions may cause human infertility. Therefore, negative results in rodent studies that are limited to only fertility and pregnancy outcomes provide insufficient information to conclude that the test substance poses no reproductive hazard in humans.

Table 10.27 lists various approaches for detecting altered male reproductive function.

TABLE 10.24
Endocrine Control of Testicular Function in Adults

Hormone	Source	Major Target	Direct or Indirect Effect on Target(s)
LH	Anterior pituitary	Leydig cells	Stimulate steroidogenesis (testosterone production)
Follicle stimulating hormone (FSH)	Anterior pituitary	Sertoli cells	Stimulate protein synthesis (e.g., androgen-binding protein)
		Sertoli and/or germ cells	Maturation of spermatids into spermatozoa (spermiogenesis)
Testosterone	Leydig cells	Male accessory glands	Maintain structure and function
		Hypothalamus and pituitary	Negative feedback control on release of FSH and LH
Estradiol	Leydig cells	Anterior pituitary	Negative feedback control on release of FSH and LH
Inhibin	Sertoli cells	Anterior pituitary	Negative feedback control on release of FSH

Source: Overstreet, J.W. and Blazak, W.F., *Am. J. Ind. Med.*, 4, 5, 1983. With permission.

TABLE 10.25
Meiotic Stages during Oogenesis and Spermatogenesis

Division	Oogenesis	Spermatogenesis
First		
Prophase I		Primary spermatocytes are formed after several mitotic divisions at puberty; the first meiotic division takes 12–14 days and occurs in the testis; spermatogenesis is an ongoing process with no “meiotic arrest”
Leptotene	Occurs during fetal development	
Zygotene		
Pachytene		
Diplotene	“Meiotic arrest” until after puberty	
Metaphase I	Preovulatory changes	Second
Anaphase I		
Teleophase I		Secondary spermatocytes
Prophase II		
Metaphase II	Ovulation	Meiosis is completed in 24 days and results in four haploid spermatids
Anaphase II	Fertilization	
Teleophase II		

Source: Dean, J., *Am. J. Ind. Med.*, 4, 31, 1983. With permission.

TABLE 10.26
Species Variability in Parameters Involving Spermatogenesis^a

Parameter	Mouse	Rat	Hamster	Rabbit	Dog	Monkey	Human
Spermatogenesis duration (days)	26–35	48–53	35	28–40	—	—	74
Duration of cycle of seminiferous epithelium (days)	8.6	12.9	—	10.7	13.6	9.5	16
Life span of							
B-type spermatogonia (days)	1.5	2.0		1.3	4.0	2.9	6.3
L + Z spermatocytes (days)	4.7	7.8		7.3	5.2	6.0	9.2
P + D spermatocytes (days)	8.3	12.2		10.7	13.5	9.5	15.6
Golgi spermatids (days)	1.7	2.9		2.1	6.9	1.8	7.9
Cap spermatids (days)	3.5	5.0		5.2	3.0	3.7	1.6
Testis weight (g)	0.2	3.7	1.8	6.4	12.0	4.9	34.0
Daily sperm production							
Per gram testis ($\times 10^6$)	54	14–22	22	25	20	23	4.4
Per individual ($\times 10^6$)	5–6	80–90	70	160	300	1100	125
Sperm reserve in cauda at sexual rest ($\times 10^6$)	49	440	575	1600		5700	420
Sperm storage in epididymal tissue ($\times 10^6$)							
Caput	20		200				
Corpus	7		175				
Cauda	40–50	300	200				420
		400					
Transit time through epididymis at sexual rest (days)							
Caput and corpus	3.1	3.0		3.0	?	4.9	1.8
Cauda	5.6	5.1		9.7	?	5.6	
Transit time through epididymis at sexual rest (days)							
Caput and corpus	3.1	3.0		3.0	?	4.9	1.8
Cauda	5.6	5.1		9.7	?	5.6	3.7
Ejaculate volume (mL)	0.04	0.2	0.1	1.0	?	?	3.0
Ejaculated sperm (10^6 /mL)	5.0	?	?	150	?	?	80.0
Sperm transit time from vagina to tube	15–60 min	30–60 min		3–4 h	20 min		15–30 min

Note: ?, unknown

^a Data obtained from various sources including Altman, P.L. and Dittmer, D.S., *Biology Data Book*, 2nd edn., Vol. I, Federation of American Societies for Experimental Biology, 1972, various tables; Eddy, E.M. and O'Brien, D.A., *Toxicology of the Male and Female Reproductive Systems*, Working, P.K., Ed., Hemisphere Publishing Corp., New York, Chapter 3, pp. 31–100, 1989; Blazak, W.F., *Toxicology of the Male and Female Reproductive Systems*, Working, P.K., Ed., Hemisphere Publishing Corp., New York, Chapter 6, pp. 157–172, 1989; Zenick, H. and Clegg, E.D., *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, Chapter 10, pp. 275–309, 1989; Spector, W.S., Ed., *Handbook of Biological Data*, W.B. Saunders Company, Philadelphia, PA, 1956, various tables.

TABLE 10.27**Sites and Mechanisms of Action of Reproductive Toxicants in the Adult Male: Approaches for Detecting Altered Reproductive Function**

Site of Action	Potentially Altered Mechanisms	Evaluative Tests
Hypothalamus	Neurotransmission	None at present
	Synthesis and secretion of GnRH	Hormone assay
	Receptors for LH, FSH, steroids	Receptor analysis
Anterior pituitary	Synthesis and secretion of LH, FSH, and PRL	Hormone assay and GnRH challenge
	Receptors for GnRH, LH, FSH, and steroids	Receptor analysis
Testis	Receptors for LH and PRL on Leydig cells	Receptor analysis
		<i>In vitro</i> production and hormone assay
	Testosterone synthesis and secretion	Morphology
		Morphology
	Vascular bed, blood flow	Receptor analysis
	Blood–testis barrier	Receptor analysis
	Receptors for FSH (Sertoli cells)	<i>In vitro</i> tests
	Receptors for steroids	<i>In vitro</i> tests
	Secretion of inhibin (ABP)	Germ cell count
	Sertoli cell function	Germ cell count and % tubules without germ cells
	Death of reserve spermatogonia	
	Spermatogonial mitosis	Spermatid counts and % of tubules with luminal sperm
	Spermatocyte meiosis	
	Spermatid differentiation	Sperm morphology
	Daily sperm production	Spermatid counts and seminal evaluation
Efferent ducts	Vascular bed	Morphology
Epididymis	Resorption	Sperm maturation
	Concentration of blood constituents	Biochemical analysis
	Secretion and interconversions	Biochemical analyses
	Enzyme activity	Biochemical analyses
	Agent transfer to luminal fluid	Assay for agent
	Smooth muscle contractility	Drug response both <i>in vitro</i> and <i>in vivo</i>
	Sperm transport	Sperm in ejaculate
Ductus deferens	Smooth muscle contractility	Response to drugs <i>in vitro</i> and <i>in vivo</i>
	Sperm transport	Sperm in ejaculate
Accessory sex gland	Secretion of agent(s)	Assays for agent(s)
	Secretion of spermicidal products	Evaluate sperm motility
Semen	Presence of agent(s)	Assay for agent(s)
	Spermicidal components	Evaluate sperm motility

Source: Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, Chapter 5, p. 89, 1992. With permission.

Notes: GnRH, gonadotrophinreleasing hormone; LH, luteinizing hormone; FSH, folliclestimulating hormone; PRL, prolactin.

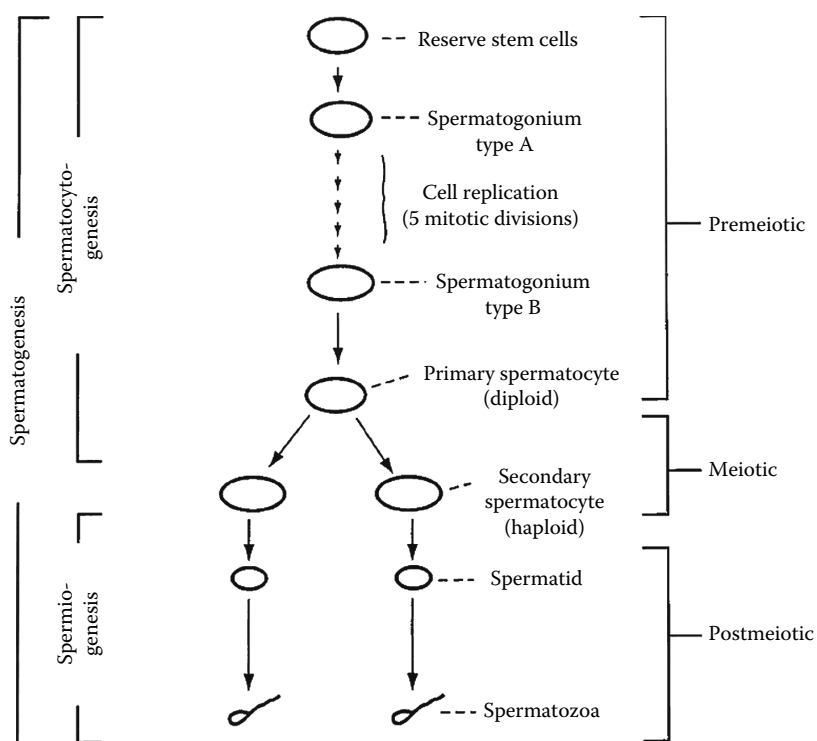


FIGURE 10.11 A general scheme of mammalian spermatogenesis, showing the premeiotic and meiotic stages of spermatocytogenesis (from reserve stem cells through the primary diploid spermatocyte to the haploid secondary spermatocyte) and the postmeiotic spermio-genesis with the development and maturation of the spermatozoa. Each cycle is completed in 35–64 days, depending on the species, with a new cycle being initiated at the type a spermatogonium level, every 12–13 days. (From Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5, p. 89. With permission.)

Female Gametogenesis

The mammalian ovulatory cycle includes multiple inter-related events involving folliculogenesis, ovulation and preparation of the reproductive tract for fertilization, and implantation leading to pregnancy. Ovulation is the central event in the ovulatory cycle.⁴⁵ Ovulation results from interaction of multiple feedback systems including the hypothalamic regions of the brain (diencephalon), anterior pituitary, and the ovary. The hypothalamus releases GnRH, and through this process regulates anterior pituitary production and secretion of gonadotropin hormones, including LH and FSH. LH and FSH released from the anterior pituitary and transported to the ovary initiate and maintain ovarian follicle growth.³⁷

The initial phase of positive feedback is that in which the hypothalamic–anterior pituitary axis component signals to initiate growth of the ovarian follicles. The mature ovarian follicle then signals to start ovulation through production and secretion of estradiol and progesterone. Estradiol and progesterone initiate a release of GnRH, which is followed by a release of the ovulation-inducing hormones, LH and FSH. These hormones provide the stimulus initiating the cascade of events in the ovary, ultimately resulting in ovulation.⁴⁵

The ovary consists of a collection of growing follicles (ova plus encasing granulosa and theca cells) lying in a dormant state in supporting tissue. The follicles arise

from a population of primordial germ cells formed during embryonic/fetal development; these germ cells undergo numerous mitotic divisions resulting in several million oogonia, the bulk of which become atretic (Figure 10.12). A few oogonia undergo meiotic reduction to the haploid state and become surrounded by a single layer of granulosa cells, this structure being the primordial follicle that remains in an arrested, meiotic, prophase state until after puberty. The other stages of meiosis will be completed after puberty and just before each ovulation. At puberty, primordial follicles are recruited into a pool of growing, primary follicles responsive to hormonal influence at the onset of each estrus cycle, one or more forming Graafian follicles and proceeding to the ovulatory follicle.⁴² Ovulation of a fertilizable ovum (oocyte, egg, or female gamete) requires formation of a corpus luteum, and growth, maturation and differentiation of three cell types. All three cell types, the germ cell (oocyte), granulosa cells, and thecal endocrine cells, are susceptible to toxic effects, as are the three major processes that occur during the development of the mature oocyte: (1) mitosis of oogonia and granulosa cells during follicular growth; (2) meiosis of oogonia to form oocytes; and (3) differentiation of granulosa cells and theca cells, allowing response to a surge of LH and subsequent ovulation.¹¹⁷ It is important to emphasize that the female mammal

carries the entire complement of ova that will be used during her breeding life span. Therefore, ovarian tissue may be affected toxicologically while *in utero* (the developing female fetus of an exposed mother) or after exposure *ex utero* at any postpartum stage of development.

Interspecies variability is the rule of thumb for female mammals as in the male, the parameters listed in Table 10.28 requiring consideration before choosing any experimental model to assess the effects of potential toxicants on female gametogenesis and preimplantation/implantation.

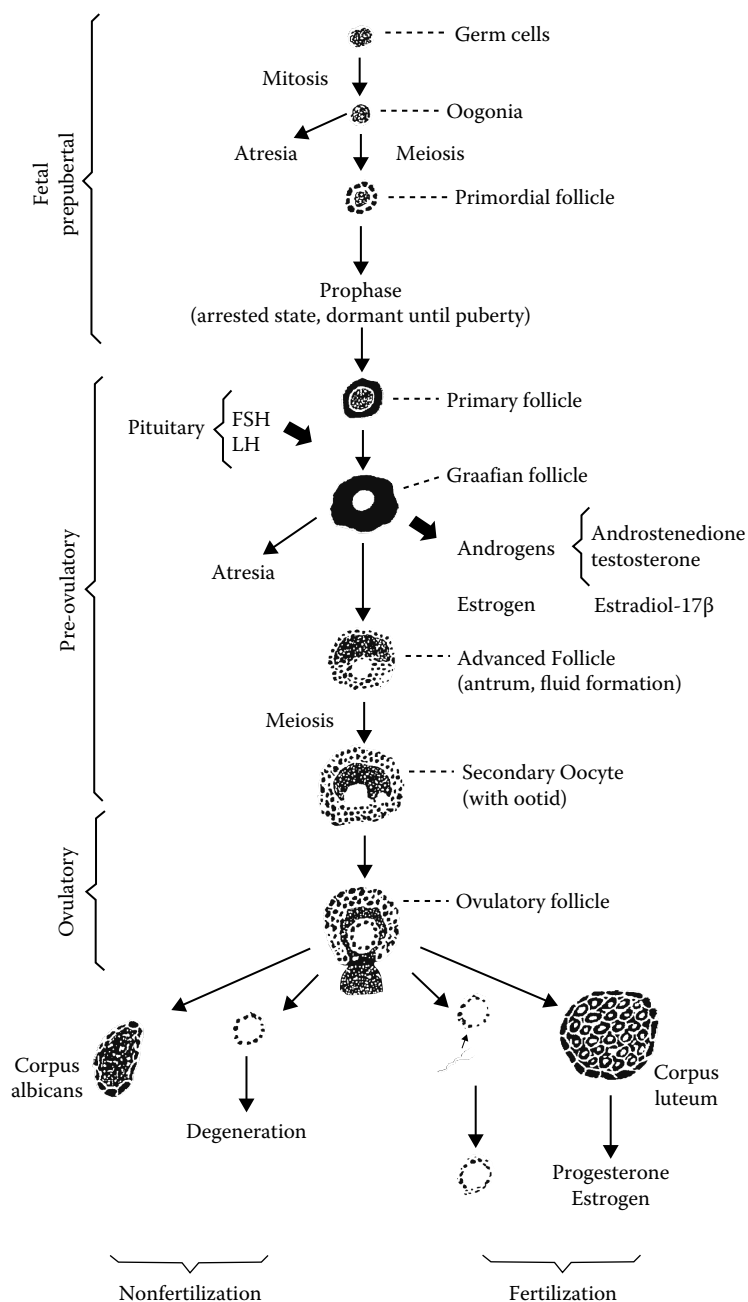


FIGURE 10.12 A general scheme of mammalian oogenesis, showing the fetal–prepubertal development of the primordial follicles that lie in an arrested state until puberty, at which time primary follicles begin to develop in response to preovulatory levels of pituitary FSH and LH, with the formation of the Graafian follicle and, subsequently, the advanced follicle that undergoes meiosis to produce a haploid oocyte. At the ovulatory stage, one mature ovum is released from each follicle. If the ovum is fertilized, the follicle becomes a steroid-secreting body, the corpus luteum, essential for the maintenance of pregnancy. If fertilization does not occur, the follicle degenerates into a mass of cells, the corpus albicans. (From Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5, p. 89. With permission.)

TABLE 10.28
Species Variability in Parameters Involving Oogenesis^a

Parameter	Mouse	Rat	Guinea Pig	Hamster	Rabbit	Cat	Dog	Monkey	Human
Sexual maturity (days)	28	46–53	84	42–54	120–240	210–245	270–425	1642	
Duration of estrus (days)	9–20 h	9–20 h	6–11 h		30	4	9	4–6	2–8
Ovulation time (days)	2–3 h	9–20 h	10 h		10 h	24–56 h	1–3	9–20	15
Ovulation type ^b	S	S	S	S	I	I	S	S	S
No. ova released	8	10	?	7	10	4–6	8–10	1	1
Follicle size (mm)	0.5	0.9	0.8		1.8		10		
Ovum diameter (mm)	0.07–0.087	0.07–0.076	0.075–0.107		0.110–0.146	0.12–0.13	0.135–0.145	0.109–0.173	0.089–0.091
Zona pellucida (mm membrane thickness)			0.012		0.011–0.023	0.012–0.115	0.135	0.012–0.034	0.019–0.035
Transport time (to reach site of implantation) (days)	4.5	3.0	3.5	3.0	2.5–4	4–8	6–8	3.0	3.0
Implantation (days)	4.5–5.0	5.5–6.0	6.0	4.5–5.0	7–8	13–14	13–14	9–11	8–13
Rate of transport of sperm to oviduct (min)	15	15–30	15		5–10				5–60
Rate of transport of embryo to uterus (h)	72	95–100	80–85		60				80
Fertile life of spermatozoa in female tract (h)	6	14	21–22		30–32				24–48
Rate of transport of ova in female tract (h)	8–12	12–14	20	5–12	6–8				24
Segmentation (to form blastocoele) (days)	2.5–4.0	4.5	5–6	3.25	3–4				5–8
Primitive streak (days)	7.0	8.5	10.0	6.0	6.5	13.0	13.0	18.0	
Duration of organogenesis (days)	7.5–16	9–17	11–25	7–14	7–20	14–26	14–30	20–45	
Gestational length (days)	20–21	21–22	65–68	16–17	31–32	58–71	57–66	164–168	

Note: ?, unknown

^a Data obtained from various sources including Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, Chapter 5, 1992; Spector, S., *Handbook of Biological Data*, W.B. Saunders Company, Philadelphia, PA, 1956, various tables; Altman, P.L. and Dittmer, D.S., *Biology Data Book*, 2nd edn., Vol. I, Federation of American Societies for Experimental Biology, 1972, various tables; Eddy, E.M. and O'Brien, D.A., *Toxicology of the Male and Female Reproductive Systems*, Working, P.K., Ed., Hemisphere Publishing Corp., New York, Chapter 3, pp. 31–100, 1989; Manson, J.M. and Kang, Y.J., *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, Chapter 11, pp. 311–359, 1989.

^b Ovulation type: I, induced; S, spontaneous.

FERTILIZATION

Fertilization occurs when the ovum meets the upward migrating sperm in the region of the ampulla of the uterine tube. The spermatozoon must penetrate the vestments of the oocyte consisting of the thick acellular zona pellucida and several layers of granulosa cells. The success of the spermatozoon requires that it reaches maturity at the appropriate time during incubation in the Fallopian tube, acquiring the capacity to fertilize the oocyte. The term capacitation is used to describe the poorly understood events leading to changes in the sperm surface and chemical changes within the cell (Table 10.29).¹²³ After capacitation, two distinct events occur: (1) the acrosome reaction, a series of fusions between specific sperm membranes, which seem to release hydrolytic enzymes,

including hyaluronidase and acrosin and may facilitate penetration of the granulosa cells; and (2) activated motility, changes in the normal motility to provide the additional propulsive thrust needed to penetrate the zone pellucida through the digested pathway. In the perivitelline space, flagellar activity of the spermatozoon ceases on contact with the oolemma, and fusion with the vitellus begins in the midregion of the sperm head followed by phagocytosis of the sperm by the ooplasm.¹²² Both capacitation and acrosome reaction are required for sperm fusion with the oolemma; these reactions are necessary even when the zona pellucida has been removed from the oocyte.¹²⁵ One can easily visualize that chemical-induced alterations in spermatozoon development at the time of capacitation, the acrosome reaction, and/or motility could influence reproductive success.

TABLE 10.29
Sperm Modification with Capacitation

Plasma membrane modifications
Changes in surface components
Loss of constituents adsorbed in excurrent ducts
Changes in antibody and lectin binding
Decrease in net negative surface charge
Changes in lipid components
Efflux of membrane cholesterol
Decrease in cholesterol/phospholipid molar ratio
Phospholipid methylation
Cleavage of sterol sulfates
Alterations in fluidity/mobility of membranes
Changes in particle distribution
Rearrangement of phospholipids
Regionalized increases in fluidity
Antigen redistribution
Increased ion permeability
Calcium
Monovalent cations
Internal modifications
Increase in intraacrosomal pH
Altered cyclic nucleotide metabolism
Increased endogenous protein carboxyl methylation

Source: Eddy, E.M. and O'Brien, D.A., *Toxicology of the Male and Female Reproductive Systems*, Working, P.K., Ed., Hemisphere Publishing Corp., New York, 1989, Chapter 3, pp. 31–100. With permission.

MIGRATION IN UTERO

With successful fertilization of the ovum in the upper end of the uterine tube, the new embryo moves downward into the uterus where the myometrial smooth muscle and epithelial layers are being prepared to receive it as a consequence of the influence of progesterone secreted by the corpus luteum (Figure 10.13). By the time the fertilized ovum has reached the uterus, it has developed into the blastocyst (blastocoele), a sphere of single cells around a fluid-filled cavity in which the impregnated ovum is expanding (Table 10.30).

At this time point, the ovum adheres or implants on the uterine wall, initiating the development of a placental structure. The preimplantation interval (see Table 10.28) is variable between species, but it represents a time when

the rapidly duplicating cells may be hypersusceptible to chemical injury, the results of such damage being reflected in the absence of implantation, early embryonic death, early fetal death/reabsorption, nonviability of newborn, and/or teratogenesis. Various quantifiable end points of reproductive toxicity are listed in Table 10.31, which can be converted into the various indices described later. In addition, because the female reproductive tract is highly dependent upon neuroendocrine centers (hypothalamus, anterior pituitary) for the secretion of appropriate growth hormones essential for specific action on the postpubertal ovary, studies of the accessory organs (oviduct, uterine muscle), cellular components of the Graafian follicle, etc., should not be ignored.

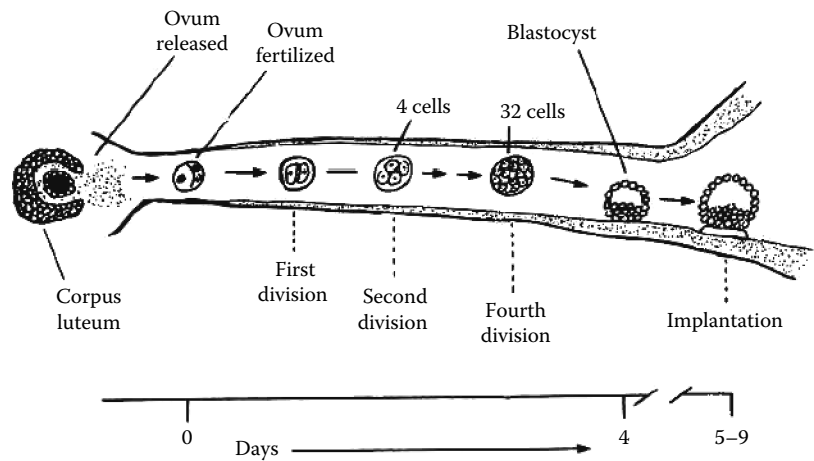


FIGURE 10.13 A schematic diagram illustrating the sequence of events after the successful fertilization of the mammalian ovum, denoting the cell divisions over the subsequent period of migration from the ampulla of the Fallopian tube to the uterus where the blastocyst begins to implant into the steroid-activated cells of the uterine muscle. The time course varies with species. (From Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5, pp. 83–112. With permission.)

TABLE 10.30
Zygote Cleavage Rates: Interspecies Comparisons^a

Parameter	Mouse	Rat	Guinea Pig	Hamster	Rabbit	Monkey	Human ^b
First cleavage spindle (h)	21–48	24–35	27–38		24		
2-Cell (h)	21–43	24–48	23–48	24–36 48–60	21–25	26–49	42–55
4-Cell (h)	38–50	48–72	30–75		25–32	24–52	
8-Cell (h)	50–64	72–96	80–82	72	32–40		90–114
16-Cell (h)	60–70	96	107	66–72	40–47		
32-Cell (h)	68–80				48	96–144	114–136

^a Spector, W.S., Ed., *Handbook of Biological Data*, W.B. Saunders Company, Philadelphia, PA, 1956, various tables.

^b Human data taken from *in vitro* culturing of inseminated (fertilized) ova; results reported in FitzGerald, L. and DiMattina, M., *Fert. Steril.*, 57, 641, 1992. With permission.

TABLE 10.31**End Points in Assessing Reproductive Toxicity**

Preconception evaluation
Mating behavior
Conception rates (preimplantation, fertilization)
Animal weight
Sperm and ovum production
Postconception evaluation
Maternal weight gain
Date of conception
Date of delivery
Implantation number
Corpora luteal number
Litter size
Deaths (embryonic, fetal)
Fetal viability (live fetuses)
Malformation incidence (external, internal)
Placental weight
Pup weight
Crown-rump length
Postnatal evaluation
Problems at parturition
Maternal–newborn relationship
Ability of dam to rear young
Postnatal growth and development
Survival incidence (day 0 to day 21)
Developmental landmarks—time of eye opening, hair growth, pinna opening, vaginal opening, etc.
Functional testing—Day 21 or later

Source: From Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5, Table 7; Haley, T.J. and Berndt, W.O., Eds., *Handbook of Toxicology*, Hemisphere Publishing Corp., Washington, DC, Chapter 7, p. 259. 1987. With permission.

REFERENCES

- U.S. Food and Drug Administration, Bureau of Foods. *Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use*, U.S. Food and Drug Administration, Washington, DC, 1966.
- U.S. Food and Drug Administration, Bureau of Foods. *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Foods*, U.S. Food and Drug Administration, Washington, DC, 1982.
- U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. *Toxicological principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Foods*, “Redbook II” (draft), U.S. Food and Drug Administration, Washington, DC, 1993.
- U.S. Food and Drug Administration. *International Conference on Harmonisation; Guideline on Detection of Toxicity to Reproduction for Medicinal Products*, Federal Register, Part IX, 59(183), 48746–48752, 1994.
- U.S. Food and Drug Administration. *International Conference on Harmonisation; Guideline on Detection of Toxicity to Reproduction for Medicinal Products; Addendum on Toxicity to Male Fertility*, Federal Register, April 5, 1996, 61(67), 1996.
- U.S. Food and Drug Administration. *International Conference on Harmonisation; Maintenance of the ICH Guideline on Toxicity to Male Fertility: An Addendum to the ICH Tripartite Guideline on Detection of Toxicity to Reproduction for Medicinal Products*, Amended November 9, 2000.
- U.S. Environmental Protection Agency. *Reproductive and Fertility Effects. Pesticide Assessment Guidelines, Subdivision F. Hazard Evaluation: Human and Domestic Animals*, Office of Pesticides and Toxic Substances, Washington, DC, EPA 540/9-82-025, 1982.
- U.S. Environmental Protection Agency. *Health Effects Test Guidelines OPPTS 870.3700, Prenatal Developmental Toxicity Study*, U.S. Environmental Protection Agency, Washington DC, EPA 712-C-96-207, pp. 1–8, 1996.
- U.S. Environmental Protection Agency. *Health Effects Test Guidelines OPPTS 870.3800, Reproduction and Fertility Effects*, U.S. Environmental Protection Agency, Washington, DC, EPA 712-C-96-208, pp. 1–11, 1996.
- U.S. Environmental Protection Agency. Guidelines for Developmental Toxicity Risk Assessment, *Federal Register*, 56(234), 63798–63826, 1991.
- Japan Ministry of Agriculture, Forestry and Fisheries. *Guidance on Toxicology Study Data for Application of Agricultural Chemical Registration*, 59 NohSan No. 4200, 45–48, 1985.
- Japan Ministry of Agriculture, Forestry and Fisheries. *Guidelines for Screening Toxicity Testing of Chemicals*, 59 NohSan No. 4200, 209–211, 1985.
- Canada, Health Protection Branch. *The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity*, Ministry of Health and Welfare, Canada, 1977.
- Drug Directorate Guidelines. *Toxicological Evaluation 2.4, Reproductive Studies*, Ministry of National Health and Welfare, Health Protection Branch, Health and Welfare, Canada, 1990.
- Committee on the Safety of Medicines (CSM). *Notes for Guidance on Reproduction Studies*, Department of Health and Social Security, Great Britain, 1974.
- World Health Organization (WHO). *Principles for the Testing of Drugs for Teratogenicity*, WHO Tech. Rept. Series, No. 364, Geneva, Switzerland, 1967.
- Organization for Economic Cooperation and Development. OECD 415: *One-Generation Reproduction Toxicity Study* (Original Guideline, adopted May 26, 1983), OECD, Paris, France, 1983.
- Organization for Economic Cooperation and Development. OECD 421: *Reproduction/Developmental Toxicity Screening Test* (Original Guideline, adopted July 27, 1995), OECD, Paris, France, 1995.
- Organization for Economic Cooperation and Development. OECD 422: *Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test* (Original Guideline, adopted March 22, 1996), OECD, Paris, France, 1996.
- Organization for Economic Cooperation and Development. OECD 414: *Prenatal Developmental Toxicity Study* (Updated Guideline, adopted January 22, 2001), OECD, Paris, France, 2001.
- Organization for Economic Cooperation and Development. OECD 416: *Two-Generation Reproduction Toxicity Study* (Updated Guideline, adopted January 22, 2001), OECD Paris, 2001.
- International Conference on Harmonization of Technical Requirements of Pharmaceuticals for Human Use. *Detection of Toxicity to Reproduction of Medicinal Products*, *Federal Register*, 59(1831), 48746–48752, 1994.

23. U.S. Food and Drug Administration. Good Laboratory Practice Regulations; Final Rule, 21 CFR Part 58, September 4, 1987.
24. U.S. Environmental Protection Agency. Toxic Substance Control Act (TSCA), Good Laboratory Practice Standards; Final Rule, 40 CFR Part 792, August 17, 1989.
25. U.S. Environmental Protection Agency. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Good Laboratory Practice Standards; Final Rule, 40 CFR Part 160, August 17, 1989.
26. U.S. Department of Health and Human Services. *Biosafety in Microbiological and Biomedical Laboratories*, 4th edn. Richmond, J.Y. and McKinney, R.W., Eds., Public Health Service Centers for Disease Control and Prevention and National Institutes of Health, Washington, DC, 1999.
27. Institute for Laboratory Animal Research, National Research Council. Guide for the Care and Use of Laboratory Animals, Washington, DC, 1996.
28. National Institute of Mental Health. *Methods and Welfare Considerations in Behavioral Research with Animals: Report of the National Institute of Mental Health Workshop*. Morrison, A.R., Evans, H.L., Ator, N.A. and Nakamura, R.K., Eds., NIH Publication No. 02-5083, U.S. Government Printing Office, Washington, DC, 2002.
29. U.S. Food and Drug Administration. International Conference on Harmonisation; Guideline on Detection of Toxicity to Reproduction for Medicinal Products, *Federal Register*, Part IX, 59(183), 48746–48752. In November 2005, the ICH incorporated the S5B addendum with S5A and retitled the combined S5 document. The contents of the two guidances were not revised.
30. Hurtt, M.E., Daston, G., Davis-Bruno, K., Feuston, M., Silva Lima, B., Makris, S., McNERney, M.E. et al. Workshop summary: Juvenile animal studies: Testing strategies and design, *Birth Defects Research (Part B)*, 71, 281–288, 2004.
31. U.S. Food and Drug Administration, CDER. Guideline for industry, Nonclinical Safety Evaluation of Pediatric Drug Products. <http://www.fda.gov/cder/guidance/5671fnl.pdf>, 2006.
32. Dietert, R.R. and Hopsapple, M.P., Methodologies for developmental immunotoxicity (DIT) testing, *Animal Models in Immunotoxicology*, 41(1), 123–131, 2007.
33. Chellman, G.J., Bussiere, J.I., Makori, N., Martin, P.L., and Ooshima, Y. Developmental and reproductive toxicity studies in nonhuman primates, *Birth Defects Research Part B*, 86(6), 446–462, December 2009.
34. Martin, P.L. and Weinbauer, G.F. Developmental toxicity testing of biopharmaceuticals in nonhuman primates: previous experience and future directions, *International Journal of Toxicology*, 29, 552–568, 2010.
35. Dietert, R.R. and Hopsapple, M.P. Methodologies for developmental immunotoxicity (DIT) testing, *Animal Models in Immunotoxicology*, 41(1), 123, 2007.
36. Wilson, J.G. *Environment and Birth Defects*, Academic Press, New York, 1973.
37. York, R.G., Parker, R.M., and Haber, L.T. Test methods for assessing female reproductive and developmental toxicology, in *Principles and Methods of Toxicology*, 6th edn., Hayes, A.W., Ed., 2012 (in Press).
38. Khera, K.S., Grice, H.C., and Clegg, D.J. *Current Issues in Toxicology: Interpretation and Extrapolation of Reproductive Data to Establish Human Safety Standards*, Springer-Verlag, New York, 1989.
39. Parker, R.M. Testing for reproductive toxicity, in *Handbook of Reproductive Toxicology*, 3rd edn., Hood, R., Ed., CRC Press, Boca Raton, FL, 2011.
40. Spector, W.S. *Handbook of Biological Data*, WADC Technical Report 56-273, W.B. Saunders, Philadelphia, PA, 1956.
41. Beck, F. and Lloyd, J.B. Comparative placental transfer, in *Handbook of Teratology*, Wilson, J.G. and Fraser, F.D., Eds., Plenum Press, New York, 1977, Chapter 5, pp. 155–183.
42. Dawes, G.S. *Foetal and Neonatal Physiology: A Comparative Study of the Changes at Birth*, Year Book Medical Publishers, Chicago, IL, 1968.
43. Ecobichon, D.J. Reproductive toxicology, in *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5, pp. 83–112.
44. Moore, K.L. *The Developing Human*, W.B. Saunders Co., Philadelphia, PA, 1993.
45. Hafez, E.S.E. *Reproduction in Farm Animals*, Lea & Febiger, Philadelphia, PA, 1968.
46. Hoar, R.M. and Monie, I.W. Comparative development of specific organ systems, in *Developmental Toxicology*, Kimmel, C.A. and Buelke-Sam, J., Eds., Raven Press, New York, 1981, p. 13.
47. O'Rahilly, R. *Development Stages in Human Embryos. Part A: Embryos of First Three Weeks (Stages 1–9)*, Publication 631, Carnegie Institute, Washington, DC, 1973.
48. Streeter, G.L. Developmental horizons in human embryos (XI–XII), *Contributions to Embryology*, 30, 211–245, 1942.
49. Streeter, G.L. Developmental horizons in human embryos (XIII–XIV), *Contributions to Embryology*, 31, 27–63, 1945.
50. Streeter, G.L. Developmental horizons in human embryos (XV–XVIII), *Contributions to Embryology*, 32, 133–203, 1948.
51. Witschi, E. *Development of Vertebrates*, W.B. Saunders, Philadelphia, PA, 1956.
52. Altmann, P.L. and Dittmer, D.S. *Growth, Federation of American Societies of Experimental Biology*, Washington, DC, 1962.
53. Hamilton, W.J. and Mossman, H.W. *Human Embryology*, 4th edn., Heffer, Cambridge, 1972.
54. Monie, I.W. Comparative development of the nervous, respiratory, and cardiovascular systems, *Environmental Health Perspectives*, 18, 55–60, 1976.
55. O'Rahilly, R. and Meucke, E.C. The timing and sequence of events in the development of the human urinary system during the embryonic period proper, *Zeitschrift für Anatomie und Entwicklungsgeschichte*, 139, 99, 1972.
56. Hendrickx, A.G. and Sawyer, R.H. Embryology of the rhesus monkey, in *The Rhesus Monkey*, Vol. 2, Bourne, G.H., Ed., Academic Press, New York, 1975, pp. 141–169.
57. Shepard, T.H. *Catalog of Teratogenic Agents*, 2nd edn., Johns Hopkins University Press, Baltimore, MD, 1976.
58. Heuser, C.H. and Streeter, G.L. Development of the macaque embryo, *Contributions to Embryology*, 29, 15–55, 1941.
59. Halman, M.T. and Prickett, M. The development of the external form of the guinea-pig (*Cavia cobaya*) between the ages of eleven days and twenty days of gestation, *American Journal of Anatomy*, 49, 351–373, 1931/31.
60. Scott, J.P. The embryology of the guinea pig. 1. Table of normal development, *American Journal of Anatomy*, 60, 397–432, 1937.
61. Edwards, J.A. The external development of the rabbit and rat embryo, in *Advances in Teratology*, Woollam, D.H., Ed., Academic Press, New York, 1968, pp. 239–263.
62. Minot, C.S. and Taylor, E. Normal plates of the development of the rabbit, in *Normentafeln Zur Entwicklungsgeschichte der Wirbelthier*, Keibel, F. and Gischer, G., Eds., Jena, 1905, p. 5.

63. Monie, I.W. Comparative development of rat, chick and human embryos, in *Teratologic Workshop Manual (Supplement)*, Pharmaceutical Manufacturers Association, Berkeley, CA, 1965, pp. 146–162.
64. Waterman, A.J. Studies of the normal development of the New Zealand White strain of rabbit, *American Journal of Anatomy*, 72, 473–515, 1943.
65. Farris, E.J. and Griffith, J.R. *The Rat in Laboratory Investigation*, 2nd edn., Haffner, New York, 1962.
66. Nelson, O.E. *Comparative Embryology of the Vertebrates*, McGraw-Hill, New York, 1953.
67. Otis, E.M. and Brent, R. Equivalent ages in mouse and human embryos, *Anatomical Record*, 120, 33–63, 1954.
68. Snell, G.D. and Stevens, L.C. The early embryology of the mouse, in *Biology of the Laboratory Mouse*, Green, E.L., Ed., Blakiston, Philadelphia, PA, 1966, pp. 205–245.
69. Boyer, C.C. Chronology of development for the golden hamster, *Journal of Morphology*, 92, 1–37, 1953.
70. Pei, Y.F. and Rhodin, J.A.G. The prenatal development of the mouse eye, *Anatomical Record*, 168, 105–126, 1970.
71. Zoetis, T. and Hurtt, M.E. Species comparison of lung development, *Birth Defects Research (Part B)* 68, 121–124, 2003.
72. Sissman, N.J. Developmental landmarks in cardiac morphogenesis: Comparative chronology, *American Journal of Cardiology*, 25, 141–148, 1970.
73. Zoetis, T. and Hurtt, M.E. Species comparison of anatomical and functional renal development, *Birth Defects Research (Part B)*, 68, 111–120, 2003.
74. Zoetis, T., Tassinari, M.S., Bagi, C., Walthall, K., and Hurtt, M.E. Species comparison of postnatal bone growth and development, *Birth Defects Research (Part B)*, 68, 86–110, 2003.
75. Marty, M.S., Chapin, R.E., Parks, L.G., and Thorsrud, B.A. Development and maturation of the male reproductive system, *Birth Defects Research (Part B)*, 68, 125–136, 2003.
76. Beckman, D.A. and Feuston, M. Landmarks in the development of the female reproductive system, *Birth Defects Research (Part B)*, 68, 137–143, 2003.
77. Holsapple, M.P., West, L.J., and Landreth, K.S. Species comparison of anatomical and functional immune system development, *Birth Defects Research (Part B)*, 68, 321–334, 2003.
78. Middle Atlantic Reproduction and Teratology Association. A compilation of terms used in developmental toxicity evaluations, 1989.
79. Wise, L. D., Beck, S. L., Beltrame, D., Beyer, B. K., Chahoud, I., Clark, R. L., Clark, R. et al. Terminology of developmental abnormalities in common laboratory mammals (Version 1), *Congenital Anomalies*, 37, 165–210, 1997.
80. Makris, S. L., Solomon, H. M., Clark, R., Shiota, K., Barbellion, S., Buschmann, J., Ema, M. et al. Terminology of developmental abnormalities in common laboratory mammals (Version 2), *Congenital Anomalies*, 49, 123–246, 2009.
81. Johnson, E.M. Perspective on reproductive and developmental toxicology, *Toxicology and Industrial Health* 2, 453–482, 1986.
82. Risk Assessment Forum, US Environmental Protection Agency. Guidelines for reproductive toxicity risk assessment, *Federal Register*, 61(212), 56274, 1996.
83. Davis, D.L., Friedler, G., Mattison, D., Morris, R. Male-mediated teratogenesis and other reproductive effects: biologic and epidemiologic findings and a plea for clinical research. *Reproductive Toxicology*, 6, 289, 1992.
84. Colie, C.F. Male mediated teratogenesis, *Reproductive Toxicology*, 7, 3, 1993.
85. Savitz, D.A., Sonnenfeld, N.L., Olshan, A.F. Review of epidemiologic studies of paternal occupational exposure and spontaneous abortion, *American Journal of Industrial Medicine*, 25, 361, 1994.
86. Trasler, J.M., Doerksen, T. Teratogen update: paternal exposures—reproductive risks, *Teratology*, 60, 161, 1999.
87. Knobil, E., Neill, J.D., Greenwald, G.S., Markert, C.L., Pfaff, D.W. *The Physiology of Reproduction*, Raven Press, New York, 1994.
88. Hood, R.D. Maternal versus developmental toxicity, in *Developmental Toxicology—Risk Assessment and the Future*, Hood, R.D., Ed. Van Nostrand Reinhold, New York, 1990, pp. 67–75.
89. Hood, R.D., Miller, D.B. Maternally mediated effects on development, in *Handbook of Developmental Toxicology*, Hood, R.D., Ed. CRC Press, Boca Raton, FL, 1997, p. 61.
90. Le Vier, R.R., Jankowiak, M.E. The hormonal and antifertility activity of 2,6-cis-diphenylhexamethylcyclotetrasiloxane in the female rat, *Biology of Reproduction*, 7, 260, 1972.
91. Sonawane, B.R., Yaffe, S.J. Delayed effects of drug exposure during pregnancy: reproductive function, *Biological Research in Pregnancy and Perinatology*, 4, 48, 1983.
92. Cummings, A.M., Gray, L.E. Methoxychlor affects the decidual cell response of the uterus but not other progesterational parameters in female rats. *Toxicology and Applied Pharmacology*, 90, 330, 1987.
93. Hood, R.D. and Parker, R.M. Reproductive and developmental toxicology, in *Preclinical Development Handbook*, Gad, S., Ed. John Wiley Press, New York, 2008.
94. Home Office Paper 5: Use of nonhuman primates in regulatory toxicology.
95. Terasawa, E.I. and Fernandez, D.I. Neurobiological mechanisms of the onset of puberty in primates, *Endocrine Reviews*, 22(1), 111–151, 2001.
96. Plant, T.M. and Marshall, G.R. The functional significance of FSH in spermatogenesis and control of its secretion in male primates, *Endocrine Reviews*, 22(6), 764–786, 2001.
97. Hendrickx, A.G. and Hummler, H. Teratogenicity of all-trans retinoic acid during early embryonic development in the cynomolgus monkey (*Macaca fascicularis*), *Teratology*, 45, 65–74, 1992.
98. Vogel, F. How to design male fertility investigations in cynomolgus monkey, in *Towards New Horizons in Primate Toxicology*, Korte, R. and Weinbauer, G., Eds., Waxmann Verlag, Münster, Germany, 2000.
99. De Kretser, D.M., Kerr, J.B. The cytology of the testis, in *The Physiology of Reproduction*, Knobil, E. and Neill, J., Eds., Raven Press, New York, 1988, p. 837.
100. Clermont, Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiological Reviews*, 52, 198–236, 1972.
101. Boorman, G.A., Chapin, R.E., Mitsumori, K. Testis and epididymis, in *Pathology of the Fischer Rat*, Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery, Jr. C.A., MacKenzie, W.F., Eds., Academic Press, San Diego, CA, 1990, p. 405.
102. Russell, L.D. Normal testicular structure and methods of evaluation under experimental and disruptive conditions, in *Reproductive and Developmental Toxicity of Metals*, Clarkson, T.W., Nordberg, G.F., Sager, P.R., Eds., Plenum Publishing Co., New York, 1983, p. 227.

103. Steinberger, E. and Steinberger, A. Spermatogenic function of the testis, in *Handbook of Physiology*, Hamilton, D.W. and Greep, R.O., Eds., Williams & Wilkins, Baltimore, MD, 1975, Vol. 5, Section F, pp. 1–19.
104. Miller, R.K., Kellogg, C.K., and Saltzman, R.A. Reproductive and perinatal toxicology, in *Handbook of Toxicology*, Haley, T.J. and Berndt, W.O., Eds., Hemisphere Publishing, Washington, DC, 1987, Chapter 7, pp. 195–309.
105. Robaire, B. and Hermo, L. Efferent ducts, epididymis and vas deferens: structure, functions and their regulation, in *The Physiology of Reproduction*, Knobil, E. and Neill, J., Eds., Raven Press, New York, 1988, Chapter 23, pp. 999–1080.
106. Russell, L.D. Normal testicular structure and methods of evaluation under experimental and disruptive conditions, in *Reproductive and Developmental Toxicity of Metals*, Clarkson, T.W., Nordberg, G.F., and Sagar, P.R., Eds., Plenum Press, New York, 1983, pp. 227–252.
107. Johnson, A.D., Gomes, W.R. The Testis. *Advances in Physiology, Biochemistry and Function*, Volume IV, Academic Press, New York, 1977.
108. Russell, L.D., Ettlin, R., Sinha-Hikim, A.P., Clegg, E.D., Eds. *Histological and Histopathological Evaluation of the Testis*, Cache River Press, Clearwater, FL, 1990.
109. Scialli, A.R. and Clegg, E.D. *Reversibility in Testicular Toxicity Assessment*, CRC Press, Boca Raton, FL, 1992.
110. Oakberg, E.F. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal, *American Journal of Anatomy*, 99, 391–413, 1956.
111. Chapin, R.E., Conner, M.W. Testicular histology and sperm parameters, in *An Evaluation and Interpretation of Reproductive Endpoints for Human Risk Assessment*, Daston, G., Kimmel, C., Eds., ILSI Press, Washington, DC, 1999, p. 28.
112. Hess, R.A. Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: light microscopic observations of perfusion-fixed and plastic-embedded testes, *Biology of Reproduction*, 43, 525, 1990.
113. Amann, R.P. A critical review of methods for evaluation of spermatogenesis from seminal characteristics, *Journal of Andrology*, 2, 37, 1981.
114. Working, P.K. Male reproductive toxicity: Comparison of the human to animal models. *Environmental Health*, 77, 37, 1988.
115. Aafjes, J.H., Vels, J.M., Schenck, E. Fertility of rats with artificial oligozoospermia. *Journal of Reproductive Fertility*, 58, 345, 1980.
116. Meistrich, M.L. Quantitative correlation between testicular stem cell survival, sperm production, and fertility in the mouse after treatment with different cytotoxic agents, *Journal of Andrology*, 3, 58, 1982.
117. Robaire, B., Smith, S., Hales, B.F. Suppression of spermatogenesis by testosterone in adult male rats: effect on fertility, pregnancy outcome and progeny, *Biology of Reproduction*, 31, 221, 1984.
118. Overstreet, J.W. and Blazak, W.F. The biology of human male reproduction: an overview, *American Journal of Industrial Medicine*, 4, 5–15, 1983.
119. Dean, J. Preimplantation development: biology genetics and mutagenesis, *American Journal of Industrial Medicine*, 4, 31–49, 1983.
120. Altman, P.L. and Dittmer, D.S. *Biology Data Book*, 2nd edn., vol. 1, Federation of American Societies for Experimental Biology, Bethesda, MD, 1972.
121. Eddy, E.M. and O'Brien, D.A. Biology of the gamete: maturation, transport, and fertilization, in *Toxicology of the Male and Female Reproductive System*, Working, P.K., Ed., Hemisphere Publishing, Washington, DC, 1989, Chapter 3, pp. 31–100.
122. Blazak, W.F. Significance of cellular endpoints in assessment of male reproductive toxicity, in *Toxicology of the Male and Female Reproductive Systems*, Working, P.K., Ed., Hemisphere Publishing, Washington, DC, 1989, Chapter 6, pp. 157–172.
123. Zenick, H. and Clegg, E.D. Assessment of male reproductive toxicity: a risk assessment approach, in *Principles and Methods of Toxicology*, Hayes, A.W., Ed., Raven Press, New York, 1989, Chapter 10, pp. 275–309.
124. Manson, J.M. and Kang, Y.S. Test methods for assessing female reproductive and developmental toxicology, in *Principles and Methods of Toxicology*, 2nd edn., Hayes, A.W., Ed., Raven Press, New York, 1989, Chapter 11, pp. 311–359.
125. Yanagimachi, R. Mechanisms of fertilization in mammals, in *Fertilization and Embryonic Development In Vitro*, Mastroianni, L., Jr. and Biggers, J.D., Eds., Plenum Press, New York, 1981, Chapter 5, pp. 81–182.
126. Haley, T.J. and Berndt, W.O., Eds. *Handbook of Toxicology*, Hemisphere Publishing, New York, 1987.

11 Juvenile Toxicology

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INTRODUCTION

Juvenile toxicology is complicated due to the dynamic, anatomic, and physiological changes that occur during growth and development of an individual. Nonclinical juvenile toxicity studies are logistically complicated due to the design of the study and their size, the number, diversity, and interdependence of end points based on target organ development, the myriad modes of actions that need to be evaluated, and the constraints due to the physical size of the animal (e.g., route of administration, necropsy techniques, blood collection, behavioral) and its growth during the study. Juvenile toxicity studies aim at bridging potential data gaps (Figure 11.1) between the pre- and postnatal toxicity studies (Segment III) where the offspring are potentially exposed *in utero* and through maternal milk until weaning and the repeat-dose toxicity studies (in young adult animals). The decision on whether

studies in juvenile animals will be required will be made by the appropriate regulatory agency. The likelihood of having to perform a juvenile toxicity study can be roughly determined by several factors: age of the pediatric patient, target organs (especially developing organs) based on clinical and nonclinical study results in adults, class history of the drug, and the indication (e.g., is it life-threatening?) (Figure 11.2). There is no STANDARD juvenile toxicity study design; however, the study designs are becoming more standardized. The study design needs to consider different stages and pace of development of the organ systems at risk in human and the animal species' equivalent. Species, age at initiation and duration of treatment, "within" versus "between" litter designs, ages at various assessments, and end points measured during and after treatment are all major considerations in designing studies on a case-by-case basis.

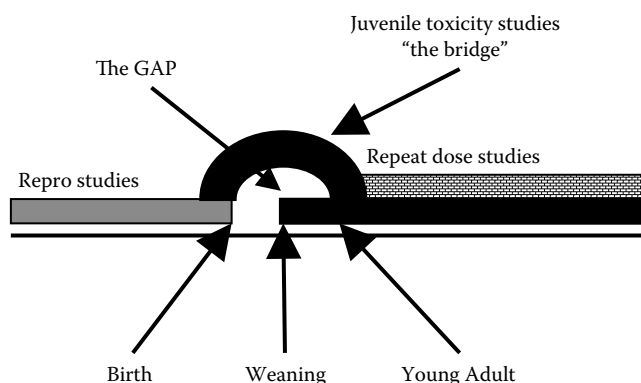


FIGURE 11.1 Juvenile toxicity studies aim at bridging potential data gaps between the pre- and postnatal toxicity studies (Segment III; offspring potentially exposed until weaning) and the repeat-dose toxicity studies (in young adult animals). (Adapted from Lewis, E.M., De Schaepdrijver, L.M. and Coogan, T.P. in: Hoberman, A.M. and Lewis, E.M., eds., *Pediatric Non-Clinical Drug Testing*, Chapter 14, p.26, John Wiley & Sons, Hoboken, NJ, 2012.)

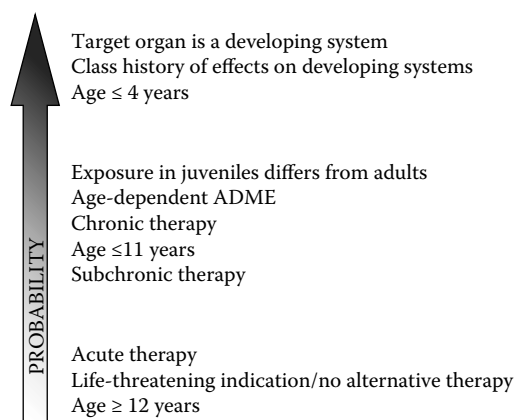


FIGURE 11.2 Probability of requiring juvenile toxicology studies. (Adapted from Laffan, S.B. and Posobiec, L., Approaches to rat juvenile toxicity studies and case studies: A pharmaceutical perspective, in: Hoberman, A.M. and Lewis, E.M., eds., *Pediatric Non-Clinical Drug Testing*, Chapter 14, John Wiley & Sons, Hoboken, NJ, 2012.)

GENERAL CONSIDERATIONS REGARDING THE NEED FOR STUDIES IN JUVENILE ANIMALS

DIFFERENCES IN DRUG SAFETY PROFILES BETWEEN MATURE AND IMMATURE SYSTEMS

Some therapeutics have shown different safety profiles in pediatric and adult patients. Inherent differences between mature and immature systems introduce the possibility of drug toxicity, or resistance to toxicity in immature systems that are not observed in mature systems. Several factors contribute to these potential differences. Postnatal growth and

development can affect drug disposition and action. Examples include developmental changes in metabolism (including the maturation rate of Phase I and II enzyme activities), body composition (e.g., water and lipid partitions), receptor expression and function, growth rate, and organ functional capacity. These developmental processes are susceptible to modification or disruption by drugs.

Although some age-dependent effects can be largely predicted by knowledge of the changes in drug metabolic pathways during development, others cannot. There are several examples of drugs that exhibit differences in toxicity between adult and pediatric patients (Table 11.1).

TABLE 11.1

Drugs that Exhibit Differences in Toxicity between Adult and Pediatric Patients

Drug	Effect	Cause	Reference
Acetaminophen	Young children are far less susceptible to acute acetaminophen toxicity than adults	Children possess a greater rate of glutathione turnover and more active sulfation resulting in a greater capacity to metabolize and detoxify an overdose than adults	Insel ¹⁴⁹
Valproic acid	Young children treated with valproic acid appear disproportionately vulnerable to fatal hepatotoxicity	Young children have a lesser capacity to metabolize and detoxify an overdose than adults	Dreifuss et al. ¹⁵⁰
Chloramphenicol	Mortality in newborns	In newborns, the exposure is increased due to a longer half-life ($t_{1/2} = 26$ h) compared to adults ($t_{1/2} = 4$ h)	Kapusink-Uner et al. ¹⁵¹
Inhaled corticosteroids	Decreased growth velocity in children	Only affects growing children which could not be determined in adult clinical trials or in nonclinical studies with mature animals	FDA Talk Paper ¹⁵²
Aspirin	Increased risk of developing Reye's syndrome in children with influenza or varicella infections	Aspirin should not be used to treat children with influenza or varicella infections, a complication not seen in adults	Belay et al. ¹⁵³
Lamotrigine	Children are at greater risk for developing hypersensitivity-type reactions, including Stevens–Johnson syndrome	Complication not observed in adults	Guberman et al. ¹⁵⁴

Source: Adapted from U.S. Food and Drug Administration (U.S. FDA), Center for Drug Evaluation and Research (CDER), Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, February 2000.

VULNERABILITY OF CHILDREN TO DRUGS AND ENVIRONMENTAL TOXICANTS

When it comes to the issue of the vulnerability of children to drugs and environmental toxicants, only the facts, impeccable science, and more research will place the field of the effects of drug and environmental toxicants on children into proper perspective. The facts clearly indicate that children are different than adults. However, few generalizations about children's vulnerability to environmental exposures apply, given that vulnerability and sensitivity are specific to a child's developmental stage, as well as being *agent-specific*.

Data obtained from toxicology studies in newborn and young animals as well as human experience with infants, children, and adolescents demonstrate that there is little scientific support for deciding that infants, children, and adolescents are, for example, consistently 10 times more sensitive to drugs and chemicals than adults. For most drugs and environmental toxicants, the no observable adverse effect level (NOAEL) is unknown. And those agents with genotoxic potential have no threshold or NOAEL. Each agent has to be individually studied to determine the toxicokinetics and dynamics for that agent.

It is also important to note that children and adolescents have better recuperative capacities than adults for many toxic agents, and similarly, appropriate drug dosages may be lesser or greater on a mg/kg or surface area basis in children than in adults in order to attain effective therapeutic blood levels or to avoid toxicity. In addition, effects produced by drugs, chemicals, and physical agents are not always deleterious nor are they always irreversible. This means that for some exposures, the young can recover from some effects more rapidly and completely than adults. If the exposure *does* result in irreversible effects by exceeding the threshold exposure, then the impact on a developing organism can be more severe than in the adult.

The child who has sustained brain damage from an infection, a stroke, or other types of brain injury may regain more function than an adult who sustains the same damage. Some anesthetics are unable to anesthetize newborn animals at exposures than to anesthetize adults, while ether alters reflexes at lower concentrations in newborn animals than in adults. Newborn mice and other animal species have demonstrated a tolerance to hypoxic conditions that is not present in adult animals, and newborn mice continue to breathe for a longer period when exposed to ether than adult mice. Newborn mice also have a prolonged survival when compared to adults when asphyxiated as a result of exposure to CO, HCN, CO₂, H₂, and CH₃. Longer exposures to strychnine, curare, CN injection, strangulation, hypoxia, or nitrobenzol are necessary to produce respiratory arrest in newborn mice as compared to adult mice. Another example where infant animals are protected relative to adults is thiourea, which is 50–400 times as toxic in the adult as in infant rats.

On the other hand, animal experiments with chloramphenicol clearly demonstrate that this drug is more toxic in the infant rat than in the adult, providing animal toxicity studies that corroborate the toxicity reported in human infants. The newborn or infant animal is more sensitive to many drugs (e.g., morphine, some other opiates, picrotoxin, tetracycline, novobiocin, some organophosphate anticholinesterases, atropine, histamine, sodium salicylate) and less sensitive to others (e.g., ethanol, strychnine, metrazol, codeine, acet-cycloheximide, thiourea, thyroid hormone). Many other drugs have sensitivities that were similar in the neonate and adult animal. There are many infections that produce more morbidity in adults than in children (e.g., hepatitis, varicella, and poliomyelitis). Drugs may also be more toxic or result in idiosyncratic effects in adults that occur rarely in children. For example, INH produces hepatitis and methotrexate produces cirrhosis more frequently in adults.

Not only are adults sometimes more vulnerable than children, adolescents can be more vulnerable than infants and children. For example, there are a number of studies that indicate that numerous radiological (x-ray) examinations increase the adolescent's risk of developing breast cancer later in life. One might expect that infants would be more susceptible to radiation-induced breast cancer than adolescents. However, the developing and proliferating adolescent breast appears to be more sensitive to radiation-induced oncogenesis than the infant breast.

It is understandable that developing organisms will have a greater sensitivity or effect from environmental toxicant exposures in many instances because

1. There is a larger proportion of cells in these organisms that are dividing and differentiating and therefore *may* result in toxic effects at lower exposures.
2. Higher exposures can occur in infants, children, and adolescents than adults, because of their behavior, not necessarily their physiology or metabolism:
 - a. Breast-feeding
 - b. Infants and children are exposed to higher quantities of toxicants because of pica, crawling, placing foreign objects in their mouth, eating dirt, exposure at day-care centers
 - c. Differences in the ratio of vital capacity-to-tidal volume; ability to metabolize or detoxify chemicals or drugs; changing relationship to weight and surface area at different ages; inefficiency of urinary clearance and excretion in newborns and infants
 - d. Differences in behavior because of psychological immaturity or peer pressure. Examples are excessive risk taking, binge drinking with lethal results, early onset smoking, unprotected sex
 - e. Variability related to differences in nutrition, healthcare, child-rearing practices, child-care practices

The events that occur during childhood and adolescent development that can be affected by drugs, chemicals, and physical agents include the following:

1. *Interference with growth, epiphyseal development, and epiphyseal closure.* Alterations in growth from exposures during development before epiphyseal closure can result in growth acceleration and growth retardation. Animal models can be used to determining the most vulnerable period during development; however, the rat does not have epiphyseal closure during the juvenile period and therefore is not an acceptable species although rabbits, dogs, and minipigs are acceptable models for epiphyseal closure.
2. *Reproductive, fertility, endocrine, and hormonal effects.* Drugs, radiation, organochlorine compounds, iodine deficiency, and other environmental exposures to endocrine-disrupting chemicals can alter estrogen, androgen, and thyroid functions thereby altering growth, timing of puberty, and maturation of sexual organs including breast development, estrous cyclicity, mating behavior, and spermatogenesis and oogenesis which may decrease fertility or cause pre- and postimplantation losses.
3. *Alteration of the adequacy of the adult immune system.* Holladay and Smialowicz¹ stated that "The possibility that developmental exposure to immunotoxicants may play a role in inducing or exacerbating hypersensitivity or autoimmune responses needs to be investigated in laboratory animals." The FDA and the EMA are currently developing guidelines for testing developmental immunotoxicity (DIT).

There is a need for special attention on DIT based on the evidence that the developing immune system may have unique susceptibilities when compared to the adult immune system. But understanding the full extent of this susceptibility has been complicated by a paucity of data detailing the development of the immune system during critical life stages as well as by the complexities of comparisons across species. There are differences between humans and the nonhuman species used in toxicity testing. These differences include specific differences relative to the timing of the development of the immune system as well as more general anatomic differences. Understanding how the timing of the immune development impacts on various immune parameters is critical to the design of juvenile toxicity studies that have immunologic assessments. Other factors important are the recognition that effects other than suppression (e.g., allergy and autoimmunity); the need to improve the assessment of DIT; and the role that histopathology has played in nonclinical juvenile toxicity studies. Pathology evaluations have been a major component of standard nonclinical toxicology studies, and could serve

an important role to evaluate juvenile toxicity studies that have immunologic assessments. For a further discussion of this topic, the reader is referred to an excellent review authored by Burns-Naas et al.²

4. *Neurobehavioral and neurological effects.* There are significant difficulties in performing these studies in animals and applying the results to humans. Precise information that equates various stages of prepartum and postpartum brain development in the human and animal models is lacking. Many of the most important neurological diseases in animal models, example, attention deficit hyperactivity disorder (ADHD), dyslexia, autism, schizophrenia, Parkinson's disease, etc., are limited. Adams et al.³ specifically discussed the topic of the "Vulnerability during the adolescent period of development." They indicate that the brain of the adolescent undergoes "striking" transformations, which is observed in many mammalian species.

Studies in juvenile animals may be useful in the prediction of age-related toxicity in children, as shown in the following examples:

- The effects of phenobarbital on cognitive performance in children were predicted by experimental studies examining the effects of this drug on the developing rodent nervous system.⁴⁻⁶
- The vulnerability of human neonates to hexachlorophene neurotoxicity was modeled in developing rats and monkeys.⁷
- The increased susceptibility of infants to verapamil-induced cardiovascular complications would be expected based on animal studies demonstrating a greater sensitivity of the immature heart to calcium channel blockade.^{8,9}
- An increased risk of convulsions in young children treated with theophylline was predicted by studies of the preconvulsant effects of this agent in developing rodents.^{10,11}

Examples of drug-induced postnatal developmental toxicity demonstrated in animals include the following:

- Neurobehavioral impairment in adult rats following early postnatal exposure to methamphetamine¹²
- The effects of methylphenidate on growth and endocrine function in young rats^{13,14}
- Apoptotic neurodegeneration in neonatal rats treated with NMDA receptor antagonists¹⁵
- Decreased myelination and axonal damage induced in preweaning rats by vigabatrin¹⁶
- Long-term changes in serotonergic innervation in rats exposed to fluoxetine during early juvenile life¹⁷
- Chondrotoxicity in immature animals treated with fluoroquinolones¹⁸

5. *Cancer*. A publication and statement made at a 2003 National Council on Radiation Protection and Measurements meeting by Eric Hall, one of the coauthors of the Brenner et al.¹⁹ paper that children are 10–15 times more sensitive to the oncogenic effects of radiation than adults. “It is clear that children are 10 times more sensitive than adults to the induction of cancer.”²⁰ Exposure can come from several sources (e.g., nuclear fallout, treatment of thyroid nodules with radioactive iodine, secondary cancers in previously treated cancer survivors, computed tomography [CT] scans for children).

UTILITY OF STUDIES IN JUVENILE ANIMALS

Adult clinical data can provide useful information regarding study design and dose selection for further study in children in some circumstances. Nonclinical developmental toxicity studies have traditionally focused on prenatal development, with only limited assessment of postnatal developmental effects. Animals used in multiple-dose toxicity studies are usually peripubertal. In some circumstances, data generated from these studies may provide sufficient information to support pediatric clinical trials without additional animal studies, particularly if the intended use includes adolescents but not younger children or infants. Since young animals in general exhibit developmental characteristics similar to pediatric patients, they are considered appropriate models for assessing drug effects in this population. The FDA believes that data from juvenile animal studies can contribute to the assessment of potential drug toxicity in the pediatric population, and can provide information that might not be derived from standard toxicology studies using adult animals, or safety information from adult humans.

It is thought that organ systems at highest risk for drug toxicity are those that undergo significant postnatal development. Thus, evaluation of postnatal developmental toxicity is a primary concern. The structural and functional characteristics of many organ systems differ significantly between children and adults as a result of the growth and development that takes place during postnatal maturation. Examples include the following:

- Brain, where neural development continues through adolescence²¹
- Kidneys, where adult levels of function are first reached at ~1 year of age²²
- Immune system, where adult levels of IgG and IgA antibody responses are not achieved until about 5 and 12 years of age, respectively²³
- Reproductive system, where maturation is not completed until adolescence²⁴
- Skeletal system, where maturation continues well into adulthood for 25–30 years²⁵

- Gastrointestinal (GI) systems, which may have direct consequences on bioavailability, clearance, and biotransformation of drugs, are functionally mature by about 1 year of age²⁶

PHARMACOKINETICS

The impact of developmental changes (e.g., somatic growth, neurobehavioral maturation, and reproduction) in drug disposition is mainly related to changes in body composition (e.g., body water content, plasma protein concentrations) (Figure 11.3) and function of organs important in metabolism (e.g., the liver) and excretion (e.g., the kidney). During the first decade of life, these changes are dynamic and often can be nonlinear and discordant making standardized dosing (using adult dosage in mg/kg) inadequate for effective drug dosing across the span of childhood. Consequently, “standard dosing” of many drugs during rapid phases of growth/development where both drug disposition and response may be markedly different is generally inadequate for the purpose of optimizing drug therapy. Selection of the appropriate dosage can only be achieved through fundamental and integrative understanding of how ontogeny influences pharmacokinetics and pharmacodynamics.²⁷

Developmental pharmacokinetics must take into account normal growth and developmental pathways.^{28,29} Increased understanding of the numerous physiological variables regulating and determining the fate of drugs in the body and their pharmacological effects has significantly improved both the efficacy and the safety of drug therapy for neonates, infants, children, and adolescents.^{30,31} The pharmacokinetics of a given drug is dependent upon age-related changes in the body composition and the acquisition of function of organs and organ systems that are important in determining drug metabolism, transport, and excretion.^{32,33} Although pediatric patients are often categorized on the basis of postnatal age (e.g., newborn infants, infants, children, and adolescents) for the study and provision of drug therapy, changes in physiology that characterize development may not correspond to these age-defined breakpoints and are not linearly related to age. In fact, during the first 12–18 months, the acquisition of organ function is most dynamic because this period is when the most dramatic changes in drug disposition occur.^{30,31} Additionally, independent from developmental changes, the pharmacokinetics of a given drug may be altered in pediatric patients also due to extrinsic (e.g., acquired diseases, comedication, diet), intrinsic, and/or (e.g., inherited diseases, genotype) factors that may occur during the first months and years of life.^{34–39} In principle, however, nonpediatric populations such as adult geriatric patients also have these same factors. To study pediatric pharmacokinetics, it is very useful to examine the impact of development on those physiological variables that govern drug absorption, distribution, metabolism, and excretion.^{28,29,32}

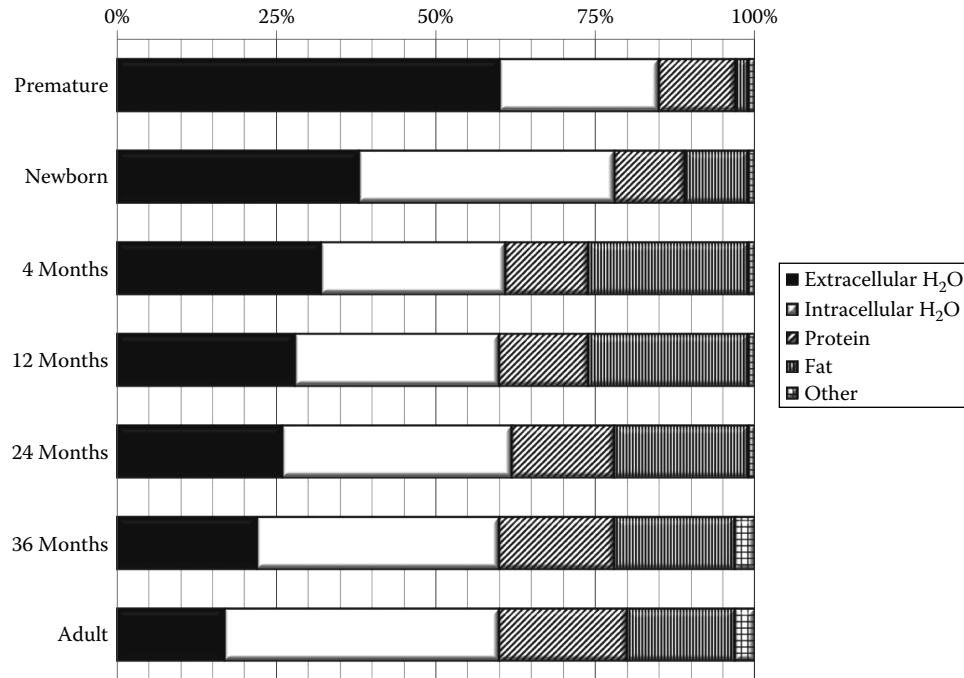


FIGURE 11.3 Ontogeny of body composition. (Adapted from Kaufman, in: Yaffe and Aranda, eds., *Pediatric Pharmacology*, pp. 212–219, 1992.)

ABSORPTION

The process of absorption for therapeutic agents administered by extravascular routes is reflected by the drug's ability to overcome barriers (e.g., chemical, physical, mechanical, biological). Developmental differences in the physiological composition and function of these barriers can change the rate and/or extent of drug absorption.^{30,31} The drug absorption (bioavailability) can be affected by developmental changes in the absorptive surfaces (e.g., GI tract, skin).^{30,31} The oral route (GI tract) is the principal means for drug administration to infants, children, and adolescents but the dermal route (skin) represents an often overlooked but important organ for systemic drug absorption as well.

GI Tract

The most important factors that influence drug absorption from the GI tract are related to the physiology of the stomach, intestine, and biliary tract. The primary differences are pH, gastric emptying, intestinal time transit time, immaturity of the intestinal mucosa leading to increased permeability, immature biliary function, high levels of intestinal β -glucuronidase activity, reduced first-pass metabolism, maturation of carrier mechanisms, and variable microbial colonization (Table 11.2).⁴⁰

Skin

The morphological and functional development of the skin as well as the factors that influence penetration of drugs into and through the skin has been reviewed by Radde and McKercher.⁴¹ Basically, the percutaneous absorption is directly related to the relative absorptive surface area and the degree of skin

hydration and inversely related to the stratum corneum thickness.⁴¹ Numerous reports describe toxicity from neonatal dermal exposure to drugs or chemicals primarily due to increased skin absorption (for example, pentachlorophenol-containing laundry detergents and hydrocortisone).^{42,43} Therefore, it is prudent to exercise extreme caution when administering topical therapy in neonates and young infants. In contrast, enhanced skin absorption of certain drugs can be used to the infant's advantage as utilized several years ago by using the percutaneous route to administer theophylline in preterm infants.⁴⁴

TABLE 11.2
Absorption (Pediatric versus Adult)

- Physicochemical properties of the drug—lipid versus water solubility
- $\uparrow V_d$ (volume of distribution)
- Small size, large surface area to body weight ratio
- Body composition (extracellular H₂O, intercellular H₂O, fat, protein)
- Gastrointestinal⁴⁰
 - Gastric pH²⁸
 - Great early postnatal variability—pH 7 (PND 0), \downarrow to pH 3 (PND 2), \uparrow to pH 7 (PND 3–13)
 - Different for premature infants—little or no free acid until PND 14
 - \uparrow Gastric pH (\uparrow basic/ \downarrow acid drug absorption)
 - Achieves adult levels around 2 year postpartum
 - \downarrow Gastric emptying for 5 month postpartum
 - Achieves adult levels around 6–8 months¹⁵⁵
- Variable GI motility
 - Infant/neonate (prolonged intestinal transit time)
 - Child (increased intestinal motility)

(continued)

TABLE 11.2 (continued)**Absorption (Pediatric versus Adult)**

- Transporters (maturation of carrier mechanisms)
 - Immature biliary function
 - ↑ Intestinal β -glucuronidase activity
 - ↓ Reduced first-pass metabolism
- Variable microbial colonization
- Skin
 - Integument—intact barrier function in full-term neonate similar to child or adolescent
 - Surface area to body weight ratio (full-term neonate)
 - ↑ Compared with older infants, children, or adolescents
 - Preterm infants—inverse correlation between permeability and gestational age¹⁵⁶
 - Permeability rates were 100- to 1000-fold greater before 30 week gestation as compared with full-term neonates
 - ↑ Permeation rate (three- to fourfold) beyond 32 weeks¹⁵⁷
 - Similar to that of full-term neonates by 2 week postpartum
- Bioavailability unpredictable
 - Topical effect is increased in neonates
 - IM absorption is increased
 - Inhalation absorption is increased

DISTRIBUTION

Drug distribution is influenced by a variety of drug-specific physiochemical factors, including drug transporters, blood/tissue protein binding, blood and tissue pH, and perfusion.^{28,30,31} However, age-related changes in drug distribution are primarily related to developmental changes in body composition, the concentration of available binding proteins, and the capacity of plasma proteins to bind drugs. Age-dependent changes in body composition can alter the drug distribution into physiological “spaces” (Table 11.3).⁴⁵ Greater extracellular and total body water spaces in neonates and young infants, coupled with a greater water/lipid ratio in adipose stores than in adults, produce reduced plasma concentrations for drugs that distribute into these respective compartments when administered in a weight-based fashion (e.g., mg/kg). Several hydrophilic drugs such as gentamicin, linezolid, and tramadol have a significantly larger volume of distribution in neonates than in infants or adults.^{46–48} The larger volume of distribution in neonates correlates with larger extracellular water content. Some lipophilic drugs associate primarily with tissue, for these drugs the influence of age on altering the apparent volume of distribution is not as readily apparent.⁴⁸ The extent of drug binding to proteins in the plasma may influence the volume of distribution of drugs.²⁸ Only free unbound drug can be distributed from the vascular space into other body fluids and to tissues where drug–receptor interaction occurs. The most

important circulating proteins responsible for this drug binding in plasma are albumin, total protein, and total globulins. The absolute concentration of these proteins is influenced by age, nutrition, and disease. Changes in the composition and amount of these circulating plasma proteins can also influence the distribution of highly bound drugs.^{28,32} A reduction in both the binding affinity and quantity of circulating plasma proteins in the neonate and young infant often produces an increase in the free fraction of drug, thereby influencing the availability of the active moiety and potentially, its subsequent hepatic and/or renal clearance. Other factors associated with development and/or disease such as variability in regional blood flow, organ perfusion, cardiac output, cell membrane permeability, and alterations in acid–base balance can also influence drug binding and/or distribution. Drug transporters can influence drug distribution because transporters can markedly influence the extent to which drugs cross membranes in the body and whether drugs can penetrate or are secreted from the target sites.

TABLE 11.3**Distribution in Pediatric Patients**

- Tissue binding differences with age (receptor, protein ↓, blood and tissue pH, and perfusion) the concentration of free drug
- Age-dependent changes in body composition⁴⁵
 - In very young infants
 - ↑ Total body water (80%–90% of the body weight)
 - ↑ Extracellular water content (45% of the body weight in neonates) versus 20% in adults
 - ↓ Fat content (10%–15% of the body weight)
 - The amount of total body water decreases to 55%–60% by adulthood
 - ↑ Volume of distribution
- ↓ Quantity/binding affinity of circulating plasma proteins—neonate and young infant
 - Albumin
 - Total protein
 - Total globulins such as α 1 acid glycoprotein
- Factors that can variably influence drug binding and/or distribution
 - Differences in regional blood flow
 - Organ perfusion
 - Cell membrane permeability
 - Changes in acid–base balance
 - Cardiac output
- Unique exposure routes
 - Placenta—in utero
 - Milk—lactational
- Transporters can influence drug distribution (e.g., ABC efflux pump P-glycoprotein, MDR1/ABCB1)
 - Show an ontogenic profile (small intestine and lung)
 - Influence movement across membranes

METABOLISM

Drug metabolism reflects the biotransformation of an endogenous or exogenous molecule by one or more enzymes to moieties, which are more hydrophilic and can be more easily excreted. While metabolism of a drug generally lessens its ability to produce a pharmacological action, it can also result in a metabolite that has significantly greater potency, and thereby, contributes to the overall pharmacological effect of the drug. In the case of prodrugs, biotransformation is required to produce the pharmacologically active metabolite. Although drug metabolism takes place in several tissues (e.g., intestine, skin, lungs, liver), hepatic metabolism has been investigated most intensively and this metabolism has been divided conventionally into two phases.^{28,30,31} Phase I hepatic metabolism usually results in modifying the therapeutic agent or xenobiotic (e.g., through oxidation) to make the molecule more polar. Phase II hepatic metabolism usually results in addition of a small molecule (e.g., glucuronide) to the therapeutic agent to make it more polar. While there are many enzymes that are capable of catalyzing the biotransformation of drugs, the quantitatively most important are the cytochromes P450 (CYP450).⁴⁹ The specific CYP450 isoforms responsible for the majority of human drug metabolism are CYP3A4/5, CYP1A2, CYP2B6, CYP2D6, CYP2C9, CYP2C19, and CYP2E1.⁵⁰

Development has a profound effect on the expression of CYP450 with distinct patterns of isoform-specific developmental CYP expression being observed postnatally (Table 11.4). Distinct patterns of isoform-specific developmental changes in drug biotransformation are apparent for many Phase I and Phase II drug-metabolizing enzymes.^{51–54} Hines⁵⁵ has categorized the development of enzymes involved in human metabolism into three main categories: (1) those enzymes expressed during whole or part of the fetal period, but are silenced or expressed at low levels within 1–2 years postpartum; (2) those enzymes expressed throughout fetal development at relatively constant levels, but are increased to some extent postnatally; and (3) those enzymes whose onset of expression can occur in the third trimester, but are substantially increased in the first 1–2 years postpartum. The first group includes CYP3A7, flavin-containing monooxygenase 1 (FMO1), sulfotransferase 1A3/4 (SULT1A3/4), SULT1E1, and may be alcohol dehydrogenase 1A (ADH1A). The second group includes CYP2A6, 3A5, 2C9, 2C19, 2D6, 2E1, and SULT1A1. The third group includes ADH1C, ADH1B, CYP1A1, 1A2, 2A 6, 2A7, 2B6, 2B7, 2C8, 2C9, 2F1, 3A4, FMO3, SULT2A1, glucuronosyltransferases (UGT), and *N*-acetyltransferase 2.^{55–57}

In addition to these *in vitro* data, during the last two decades, there has been a rapid generation of data concerning metabolism of therapeutic agents in children. *In vivo*

data have been generated mainly through two methods.^{58,59} One methodology is the use of dedicated ontogeny studies in which a probe drug (e.g., dextromethorphan or acetaminophen/paracetamol) is administered to children of various age groups or to the same children over a period of time. The other methodology in which these *in vivo* data have been developed is serendipitously over the course of industry-sponsored or investigator-initiated pediatric clinical trials, which utilize the traditional age groups, and both anticipated as well as unexpected results reveal new data about the drug-metabolizing enzymes involved.

The most important examples of studies that have resulted in clinically important insight into the ontogeny of drug metabolism are summarized in the following paragraph:

1. Midazolam plasma clearance after IV administration
 - a. Reflects hepatic CYP3A4/5 activity^{60,61}
 - b. Increases approximately fivefold (1.2–9 mL/min/kg) over the first 3 months of life⁶²
2. Carbamazepine plasma clearance
 - a. Largely dependent upon CYP3A4⁶³
 - b. Greater in children than adults,^{64–66} thereby necessitating greater weight-adjusted (i.e., mg/kg) doses of the drug to produce therapeutic plasma concentrations
3. CYP2C9 and, to a lesser extent, CYP2C19 are primarily responsible for phenytoin biotransformation.⁶⁷ Saturable phenytoin metabolism appears postnatal day (PND) ~10 demonstrating acquisition of CYP2C9 activity⁶⁸
 - a. Phenytoin half-life
 - i. ~75 h in preterm infants
 - ii. ~20 h in term infants during the first week postpartum
 - iii. ~8 h after 2 weeks postpartum
4. The most common CYP1A2 substrates used in pediatrics studies are caffeine and theophylline
 - a. Caffeine elimination *in vivo* mirrors that observed *in vitro* mediated by CYP1A2 observed by ~4 months of age⁶⁹
 - b. Formation of CYP1A2-dependent theophylline metabolites reaches adult levels by ~4–5 months of postnatal age⁷⁰
 - c. Theophylline plasma clearance in older infants and young children generally exceeds adult values⁷¹
 - d. Furthermore, in adolescent females, caffeine 3-demethylation appears to decline to adult levels at Tanner stage II relative to males where it occurs at stages IV/V, thus demonstrating an apparent sex difference in the ontogeny of CYP1A2

TABLE 11.4**Metabolism in Pediatric Patients**

- Immature liver first-pass effect is decreased
- Phase I hepatic metabolism usually modifies the molecule (e.g., through oxidation) making it more polar
 - Maturation (0–3 years)—Enzyme activity varies with P450, substrate, age
- Phase II hepatic metabolism usually results in addition of a small molecule (e.g., glucuronide) to the therapeutic agent in order to make it more polar
 - Phase II maturation (0–12 years)
- CYP450 isoforms responsible for the majority of human drug metabolism⁵⁰
 - CYP3A4/5
 - CYP1A2
 - CYP2B6
 - CYP2D6
 - CYP2C9
 - CYP2C19
 - CYP2E1
- Development of enzymes involved in human metabolism^{55–57}
 - Fetal enzymes that are silenced or expressed at low levels within 1–2 years after birth
 - CYP3A7, flavin-containing monooxygenase 1 (FMO1), sulfotransferase 1A3/4 (SULT1A3/4), SULT1E1, and maybe alcohol dehydrogenase 1A (ADH1A)
 - Fetal enzymes that are increased postnatally
 - CYP2A6, 3A5, 2C9, 2C19, 2D6, 2E1, and SULT1A1
 - Third trimester fetal enzymes that are substantially increased 1–2 years postpartum
 - ADH1C, ADH1B, CYP1A1, 1A2, 2A 6, 2A7, 2B6, 2B7, 2C8, 2C9, 2F1, 3A4, FMO3, SULT2A1, glucuronosyl transferases (UGT), and *N*-acetyl transferase 2

EXCRETION

The primary organ responsible for the excretion of drugs and their metabolites is the kidney (Table 11.5). Maturation of renal function is a dynamic process that begins early during fetal organogenesis and completes by early childhood.^{72,73} The developmental increase in glomerular filtration rate (GFR) involves active nephrogenesis that begins at 9 weeks and completes by 36 weeks of gestation.

The use of serum creatinine as a measure of renal function in children is controversial⁷⁴ because it depends on many factors and, in the first days of life in neonates, residual maternally derived creatinine may interfere with the assay.⁷⁵ However, a comparison between serum creatinine with inulin clearance in preterm infants showed a good and clinically useful correlation and has supported serum creati-

nine as an appropriate measure of GFR in preterm infants already on Day 3 of life.⁷⁶

A more direct approach to estimate the GFR is to use a marker that is freely permeable across the glomerular capillary and neither secreted nor reabsorbed by the tubulus. Markers that have been used to measure the GFR are inulin, polyfructosan S, cystatin C, ⁵¹Cr-EDTA, ¹²⁵I-iothalamate, or mannitol.^{74,77} A marker to estimate the active tubular secretion in children is *p*-aminohippuric acid.⁷⁷

Age-related changes in GFR can dramatically alter the plasma clearance of compounds with extensive renal elimination. Therefore, GFR should be considered a major determinant for age-appropriate dose regimen selection. Pharmacokinetic studies of drugs primarily excreted by glomerular filtration (e.g., ceftazidime and famotidine) have demonstrated significant correlations between plasma drug clearance and expected maturational changes in renal function.^{78,79} For example, differences in GFR between preterm and term newborns necessitate dosing intervals for tobramycin of 36–48 h and 24 h, respectively.⁸⁰ For aminoglycosides, failure to account for the ontogeny of renal function and adjust the dosing regimens accordingly can result in exposure to potentially toxic serum concentrations. Also, concomitant medications (e.g., β -methasone, indomethacin) may alter the normal pattern of renal maturation in the neonate.³⁵ For drugs with extensive renal elimination, both maturational and treatment-associated changes in kidney function must be considered and used to individualize treatment regimens in an age-appropriate fashion.

TABLE 11.5**Excretion in Pediatric Patients**

- Kidney development
 - Nephrogenesis begins at 9 weeks and completes by 36 weeks of gestation
 - In term neonates, glomerular filtration rate (GFR) is ~2–4 mL/min/kg
 - In preterm neonates, GFR 0.6–0.8 mL/min/kg
 - During the first 2 weeks postpartum, GFR increases rapidly (due to increased renal blood flow)
 - By 8–12 months of age, adult GFR values are reached
 - Tubular secretory pathways are immature at birth and gain adult capacity during the first year
- Immature kidneys (full kidney function at 2–3 years)
 - ↓ Glomerular filtration rate
 - ↓ Tubular secretion/reabsorption
 - Perfusion to the kidneys may be ↓
 - Infant/neonate: slower clearance (CL), longer half-life ($t_{1/2}$)
 - Child: rapid CL, shorter $t_{1/2}$

JUVENILE TOXICITY STUDY PRELIMINARY CONSIDERATIONS

The information required to successfully design a nonclinical juvenile toxicity study are intended patient population, compound information (e.g., mode of action, target organs, off-target information from previous work, available toxicokinetic and pharmacokinetic data), nonclinical data (including studies completed, test species, route of administration), and all clinical data. One of the most important aspects in designing these animal studies are the application of toxicokinetics and pharmacokinetics. Exposure levels should include exposures that occur in the environment or are used clinically. This data should be obtained in a highly recommended preliminary study.

The juvenile toxicity study should be rigorously designed so that the data will determine the NOAEL. The following issues need to be addressed:

PEDIATRIC INDICATION

This topic is important because it determines the age of the test subject at initiation and termination of treatment, identifies specific requirements (e.g., animal requirements, biomarkers, bleeding techniques and intervals [blood volumes], behavioral, neurological, and immunologic assessments, histopathology). In the development of the study design, the development of target organs, the ontogeny and functionality of metabolizing enzymes, and the ontogeny of various influx and efflux transporters must be considered.

DOSING ROUTES

When performing nonclinical studies, the intended clinical route of administration and dosage formulation should be used unless an alternate route of administration and dosage formulation provides greater exposure or is less invasive with adequate exposure. Assessment of toxic effects by more than one route of administration can be appropriate if the drug is intended for clinical use by more than one route of administration. When different routes are expected to result in differences in systemic and local exposure of such magnitude that occurrence of postnatal toxicity would be expected, sponsors should consider testing by multiple routes. When the intended clinical administration is intravenous, this route should be sufficient. Because the primary purpose of these studies is to identify potential hazards, small changes in exposure/distribution by route generally would not be considered important.

Since adverse effects can sometimes be related to metabolic differences between adult and juvenile animals, toxicokinetic studies can provide useful information for assisting in-study interpretation. Assessment of developmental differences in parent drug disposition and profiles of significant metabolites in juvenile animals should be made according to established guidelines (see the ICH guideline for industry S3A Toxicokinetics: Assessment of Systemic Exposure in Toxicity Studies).⁸¹

Ideally, the intended human route of administration should be used, unless studies in adult animals have indicated that an alternative route is more relevant to human use. It is recognized that practical difficulties may occur for certain routes of administration when using juvenile animals. However, the primary purpose of these studies is to identify potential safety concerns relevant to pediatric use.

DOSE SELECTION

The primary purpose of juvenile animal studies is to assess whether young animals are more sensitive to a reaction of a medicinal product than adult animals, and to identify reactions on developing organs. It is important to establish a clear dose–response relationship for adverse effects in juvenile animals, when possible. The high dose should produce identifiable toxicity (either developmental or general). Therefore, the high dose should be selected such that frank toxicity does not occur and it is recommended that doses in the lower part of the dose–response curve established in adult animals are selected. The intermediate dose should produce some toxicity so that a dose–response relationship can be demonstrated if one exists. An intermediate dose level might not be necessary in juvenile animal studies if the differences between the low and high doses are relatively small. In order to bridge the juvenile animal data to the existing adult animal data, a common dose, preferably in the low dose range (NOAEL or NOEL), should generally be included in the juvenile animal studies. In the absence of a NOAEL in the general toxicology studies, a dose range–finding study in juvenile animals is advocated together with toxicokinetic evaluations to support dose selection. The low dose should produce little or no toxicity, and a NOAEL should be identified, if possible, and should preferably result in exposure levels similar to the anticipated clinical exposure in the intended population. The FDA and EMA recommend evaluating and potentially modifying intermediate and low doses in relation to those that produce the desired pharmacodynamic effect in the test species.

TIMING OF EXPOSURE

The timing of the intended use of the drug as it relates to periods of rapid postnatal growth and development is important. If the drug is intended for use in children undergoing phases of rapid overall growth and development, it is important to evaluate an animal model undergoing a corresponding growth phase. Organ systems mature at specific times in specific species. Human-to-animal comparisons of developmental periods for the nervous, reproductive, skeletal, pulmonary, immune, renal, cardiac, and metabolic systems are presented later. These comparisons can be used as a general guide to appropriate periods of treatment to assess the development of specific systems in various animal models. Immature animals have accelerated chronological development compared to humans, which can facilitate evaluation of long-term effects following acute or chronic exposure using well-defined end points

(e.g., assessment of reproductive or nervous function). The age of the animals at initiation of dosing should be based on the postnatal development parameters of interest. It is important that the stage of development in the animals being studied be comparable to that in the intended pediatric population.

Species considerations based on the initiation of dosing: Matching the ages of the neonate and infants so that all groups are started at the same interval can be done with the rat, and may be done with the minipig. For other species, it can be difficult to obtain dams with litters (dogs) soon after birth and other species (rabbit) cannot have their litters disturbed in the first week or so after birth. However, if the initiation of treatment is postweaning (pediatric population equivalent to ~2 or greater years), then all species can be used. If treatment starts after weaning, species (rat/dog/minipig) offer the advantage of distributing the litter mates among the treatment groups where the primate provides only one pup per dam.

DURATION OF TREATMENT

Based on the observation that embryo–fetal development is especially sensitive to perturbation during organogenesis, tissues that undergo significant postnatal development in pediatric patients and juvenile animals may also have greater sensitivity to certain drug-induced toxicities than mature tissues. Organ systems identified as undergoing considerable postnatal growth and development include the nervous, reproductive, pulmonary, renal, skeletal, GI, hepatobiliary, and immune systems. Given the variable rate of postnatal development during different periods of childhood, the definition of long-term treatment can vary by pediatric population. Intended treatment of several weeks may not be considered long term in early adolescence, but might involve considerable development for the neonate given the duration of some developmental windows.

The frequency of administration should be relevant to the intended clinical use of the drug. In some cases, however, the use of dosing frequencies similar to those anticipated for clinical administration is not feasible because of technical considerations for the animal models used. Changes in frequency can be made when variables such as metabolic and kinetic differences are considered.

The duration of treatment in animals should include at least the significant periods of relevant postnatal development for the selected species. The study duration might be limited to a particular period of development if adverse reactions are expected only in an organ system with a relatively short critical period of development (e.g., kidneys, lungs). If the aim of the study is to evaluate potential long-term effects, then dosing duration should be increased relative to the intended therapeutic use. When adverse reactions are expected on systems with a long development period (e.g., brain development, bone growth, immune function, etc.), animals should be investigated up to reaching adulthood (approximately up to 13 weeks in rats and 9 months in Beagle dogs).

One approach to consider is establishing exposure and initial tolerability in a dose range–finding study followed

by a definitive study powered to assess specific concerns. Treatment-free periods designed to assess reversibility of possible adverse effects should also be considered. Inclusion of recovery periods in studies can be valuable in distinguishing acute to intermediate pharmacodynamic effects from frank developmental toxicity, and this information could influence the evaluation of potential human risk.

SPECIES

A study in juveniles from one animal species may be sufficient to evaluate toxicity end points for therapeutics that are well characterized in both adult humans and animals. Routinely, the rat is the species of choice for most juvenile toxicity studies. The species of the juvenile animal tested should be appropriate for evaluating toxicity end points important for the intended pediatric population. Traditionally, dogs have been the nonrodent species of choice. In some circumstances, however, other species may be more appropriate. For example, when drug metabolism in a particular species differs significantly from humans, an alternative species (e.g., minipigs, pigs, monkeys) may be more appropriate for testing. When determining an appropriate species, sponsors are encouraged to consider certain factors, such as the following:

- Pharmacology, pharmacokinetics, and toxicology of the therapeutic agent
- Comparative developmental status of the major organs of concern between juvenile animals and pediatric patients
- Sensitivity of the selected species to a particular toxicity

END POINTS

The selection of end points to be monitored in a juvenile animal study is critical for assessing the reactions of a medicinal product on development and growth. Juvenile toxicity studies should be designed to determine medicinal product reactions on the overall growth of the organ systems that develop postnatally (e.g., skeletal, renal, lung, neurological, immunologic, and reproductive systems). These studies should include, at a minimum, measurement of growth (e.g., serial measurements of crown-rump length, tibia length, growth velocity per unit time, or other appropriate indices), external indices of sexual maturation, body weight, physical signs, organ weights, and gross and microscopic examinations (Figure 11.4). More specific measurements can be reserved for case-specific evaluations based on the knowledge of the pharmacological or toxicological target. The more specific the concern, the more directed the study design approach can be. A more generalized screening approach may be useful if little information is available.

Clinical pathology determinations can also be useful, but they may be limited by the technical feasibility of obtaining adequate samples for analysis, particularly in the case of juvenile rodents. Should histopathologic effects occur in male and/or female reproductive organs, then the functional consequence of this finding should be investigated

(e.g., mating and fertility, semenology). An example juvenile toxicity study design with reproductive evaluation is shown in Figure 11.5. For developmental neurotoxicity assessments, well-established methods should be used to monitor key central nervous system (CNS) functions, including assessments of reflex ontogeny, sensorimotor function, locomotor activity, reactivity, and learning and memory. Modifications of existing toxicity designs or *de novo* juvenile studies should be used depending on the concerns to be addressed.

It can be helpful to determine the relationship between toxicological end points and drug exposure (e.g., predosing,

immediately postdosing, and time of peak plasma concentration). To differentiate long-term effects on development from acute effects, it might be appropriate to measure certain end points immediately before daily administration of the drug. Also, adding recovery group animals is helpful in determining whether the drug-induced effects are reversible.

The use of *in vitro* models using juvenile animal tissue or specific disease models in juvenile animals could also be considered to study target organ toxicity. The inclusion of satellite groups of animals to study the reversibility or long-term consequences of potential adverse reactions should be considered.

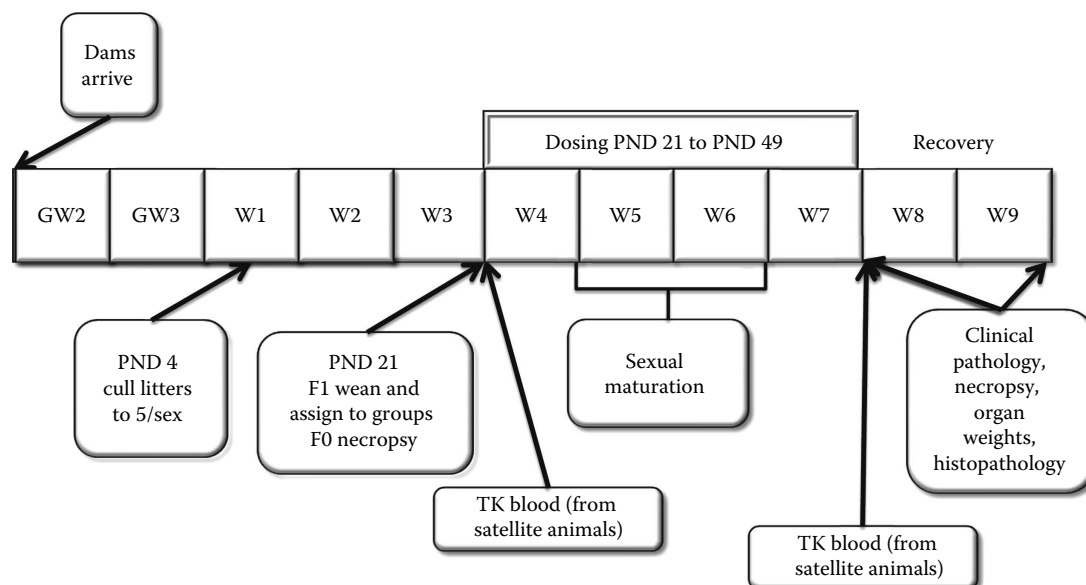


FIGURE 11.4 Juvenile toxicity study design (targeted liver).

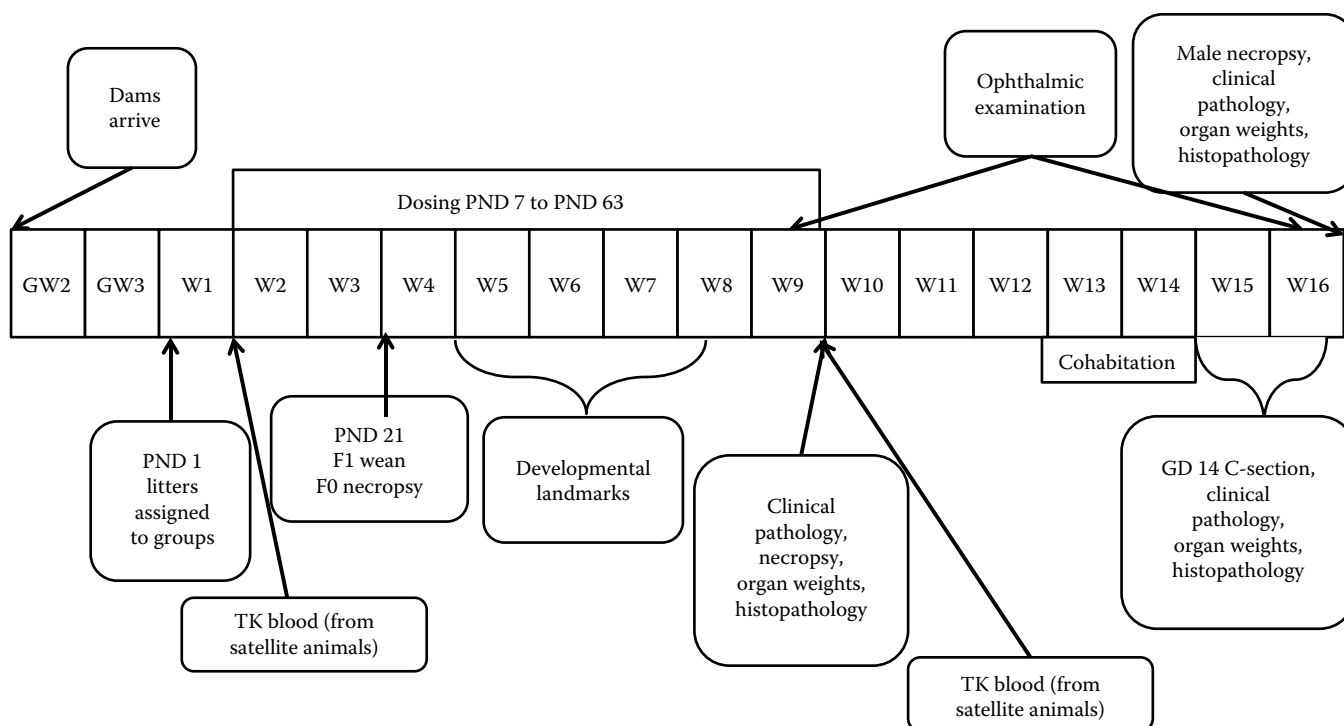


FIGURE 11.5 Juvenile toxicity study design (general toxicity with reproductive evaluation).

PRE- AND POSTNATAL REPRODUCTION STUDIES

Before performing a juvenile animal toxicity study, it should be considered whether a developmental toxicity issue could be addressed in a modified pre- and postnatal development study in rats. Key factors that need to be examined include, but are not restricted to, the amount of the active substance and/or relevant metabolites excreted via the milk and resulting plasma exposure of the pups, which period, physical development and histopathologic investigations. When a pre- and postnatal study is also being used to address a specific aspect of juvenile toxicity, such a study should be extended to include appropriate developmental end points. If specific developmental end points cannot be assessed within the context of pre- and postnatal studies, additional juvenile animal studies will be required.

NEUROTOXICITY ASSESSMENT

Neurotoxicity studies are only required if the chemical or pharmacological class of compound or previous studies in humans or animals gives cause for concern for the developing nervous system or influences for neuroendocrine system balance.

For developmental neurotoxicity assessments, where possible validated methods should be used to monitor key functional domains of the CNS, including, but not restricted to, assessments of reflex ontogeny, sensorimotor function, locomotor activity, reactivity, and learning and memory. An example juvenile toxicity study design with targeted CNS and reproductive evaluations is shown in Figure 11.6.

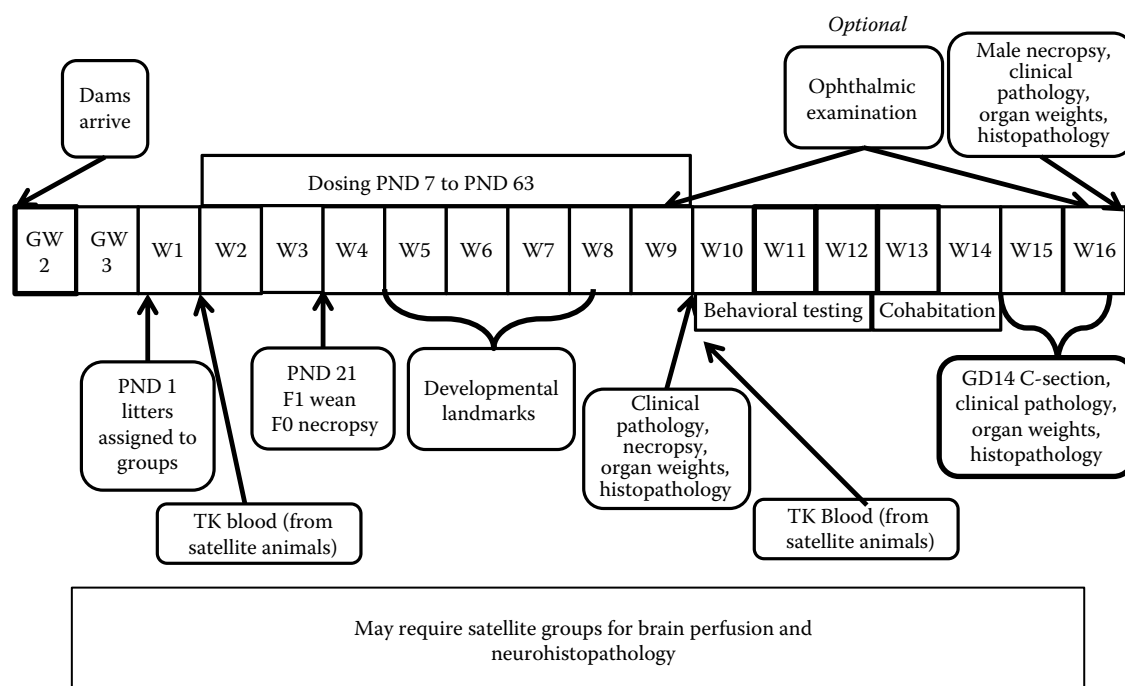


FIGURE 11.6 Juvenile toxicity study design (targeted CNS and reproductive evaluation).

IMMUNOTOXICITY ASSESSMENT

Immunotoxicity studies are only required if the chemical or pharmacological class of compound or previous studies in humans or animals gives cause for concern for the developing immune system. Pre- and postnatal exposure can potentially result in all types of immunotoxicity in the offspring (e.g., immune suppression, hypersensitivity, allergy and autoimmune disease).

A study should be based on immune assays already validated, but the experimental design should be flexible. Histopathology should be included as well as functional assays such as T-cell-dependent antibody response, host resistance assay and cell-mediated immune assay. An example juvenile toxicity study design with targeted immunotoxicity evaluations is shown in (Figure 11.7).

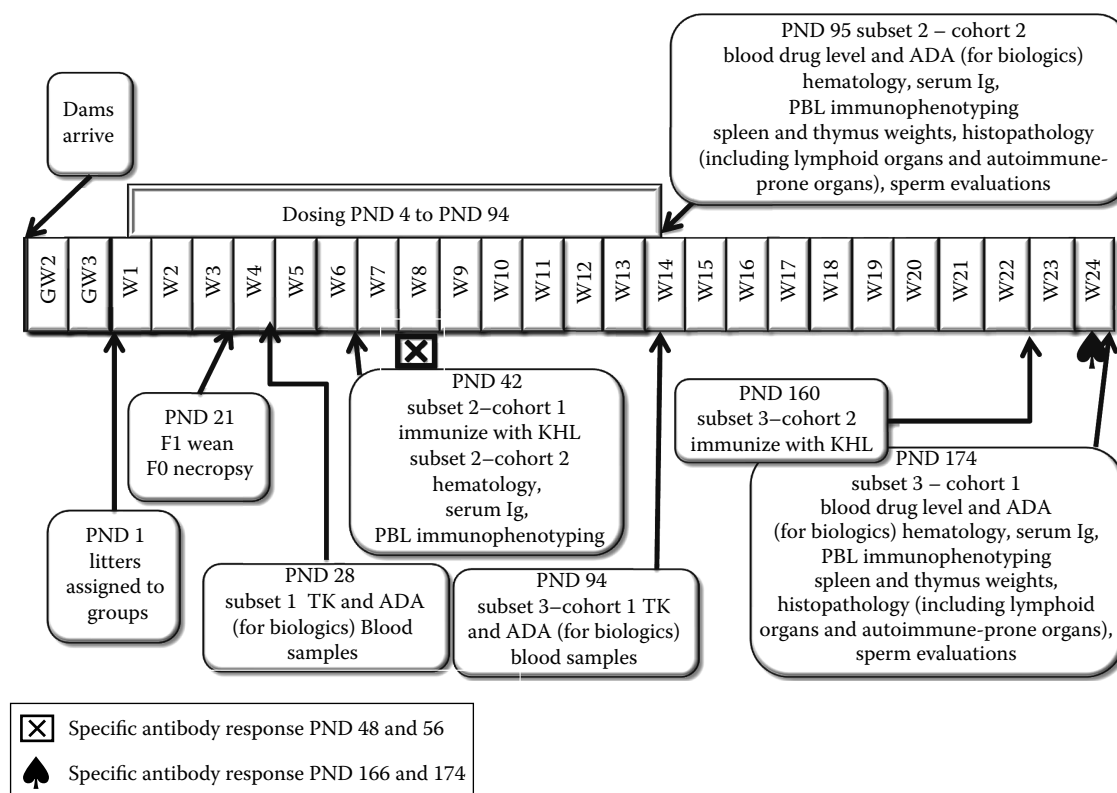


FIGURE 11.7 Juvenile toxicity study design (targeted immunologic evaluation). (Developmental immunotoxicity end points loosely adapted from Collinge, M. et al., *J. Immunotoxicol.*, 1, 2012.)

NEPHROTOXICITY ASSESSMENT

Nephrotoxicity studies are only required if the chemical or pharmacological class of compound in previous studies in humans or animals gives cause for concern for the developing renal system. For developmental nephrotoxicity assessments, where possible, validated methods should be used to monitor key functional parameters in the urine of a relevant species.

COMPARATIVE SPECIES DEVELOPMENT

NERVOUS SYSTEM

CNS Development

In a review article entitled "Species Comparison of Postnatal CNS Development: Functional Measures," Watson et al.⁸² identify key events in human CNS development and compare them, when possible, to other species (Table 11.6). Their review presented morphological and functional data relating to the postnatal development of the CNS of vertebrates with an emphasis on brain growth, neurogenesis, myelination, synaptogenesis, and neuronal and synaptic pruning.

There is general recognition that the developing nervous system is qualitatively different from the adult system. With respect to the CNS, the human is an altricial species, that is, born in a relatively immature state and must undergo considerable postnatal development to reach its adult state of maturation. A large percentage of laboratory animals, such as mice, rats, cats, dogs, rabbits, and nonhuman primates, are like humans and must also undergo a prolonged period of neurological development postnatally.

Some species, like guinea pigs and sheep, are born with very mature nervous systems, however, which undergo little CNS development postnatally. These precocial species may be of little practical use as a comparative model to the human CNS.

General features of human perinatal neurological development are fairly well known (Table 11.7). Human brain growth relative to adult weight is at its peak at birth.⁸³ During the perinatal period, brain nuclei, cortical structures, and major connections are established. By the first week of postnatal life, cortical neurogenesis and migration are considered to have been completed. Subsequent neocortical development is largely dependent on synaptic maturation or elimination, as well as on selective neuronal death.⁸⁴ From birth to 3 months of age, the transient fine organization of connections continues, as does an overproduction of synapses, and neuropeptide maturation. Cerebral hemisphere myelination continues, extending to the frontal lobe by 9 months of age, though myelination is not complete until adulthood (early 20s) (Table 11.8). Peak synapse formation continues 2–3 years of age.^{85,86}

The visual system shows major differences at birth between experimental animals and humans. Human infants are born with open eyes and a relatively well-developed visual system. In contrast, most experimental neonatal animals have closed eyes at birth. The less-developed visual system is more susceptible to agents that can potentially harm vision. For example, excess oxygen exposure to newborn rats, cats, and

dogs can cause irrevocable damage in the developing visual system; however, full-term newborn humans would not be adversely affected.⁸⁷ However, premature infants (with a less-developed visual system) treated with supplemental oxygen therapy for a number of days can develop retinopathy.⁸⁸

The postnatal neurological development in humans, rats, and nonhuman primates is extensive. The primary developmental events occurring postnatally in humans are myelination, synapse production, and neuronal and synaptic pruning. Neuronal pruning is the programmed cell death (apoptosis) in the rodent neocortex that peaks around PND 4–7, reducing the neuronal number by ~20%–30%. In addition to the loss of neurons, synapse loss occurs until early adulthood, even later than apoptosis. This loss of a large numbers of neurons and synapses is required for proper development of the CNS. Almost all of the information on neural pruning has been derived from the rodent model, there is scant information on these processes in primates.

In order for an experimental species to be predictive of toxic effects to development of the human CNS, the relevant developmental processes occurring in the human population during the period of exposure must also occur in the test species. The experimental species with the most available information pertaining to neurological development at birth is the rat. Two well-established differences exist between rat and human: (1) peak brain growth as a percentage of adult body weight occurs around PND 7–8 in the rat, while in the human it occurs at birth⁸⁹ and (2) little myelination is acquired prenatally in the rat in contrast to the human⁹⁰ (Table 11.9). Romijn et al.,⁹¹ using four parameters of brain development (synaptogenesis, development of glutamate decarboxylase and choline acetyltransferase activity, and the development of neurological electrical activity characteristic of active and quiet sleep), concluded that the rat pup at PND 12–13 is approximately equivalent of a human newborn.

In general, the same processes occur in rats. For example, maximum brain growth velocity occurs at or soon after birth in rats and humans, myelination occurs predominately postnatally, and a significant amount of synaptogenesis continues postnatally. This is not to say that postnatal development of the CNS is identical between rats and humans. In fact, there are many examples where the human is more developed at birth than the rat, such as neurogenesis in the dentate gyrus (in the human, about 20% of the granule cell population is formed after birth compared to >80% in the rat) and myelination (the peripheral nerves, the pons, and the cerebellar peduncles are fairly well myelinated in humans at birth; however, little myelin is acquired in the rat prenatally). However, because these developmental processes occur earlier in human development, the differences in maturation timelines would not be expected to impact the viability of rat as a model for potential toxicity to human postnatal brain development.

While the nonhuman primate might be considered the best model for postnatal human brain development, this is not necessarily the case. Regional differences are not uncommon. For example, the rat is a better model for developmental neurotoxicity of the cerebellum, a late developing brain region (Table 11.10). The human cerebellum reaches its peak growth rate at ~4–7

months and is ~50% adult size at 1 year. The rat has its peak growth rate from PND 5–10 and the 50% adult size landmark is achieved at PND 15. In contrast, the rhesus monkey achieves the 50% adult size landmark at birth. Therefore, peak growth occurs prenatally in the rhesus monkey, but postnatally in rat and human. However, one significant area where the monkey is more similar to humans is the synaptogenesis pattern. This pattern is unique to the primate cortex. Synapse overgrowth phase is followed by a plateau phase in synapse formation, and then a regressive phase occurs where synapses decreased in number and are being reorganized. In the human cerebral cortex, a 2 year old has approximately twice as many synapses than an adult. Synaptic proliferation and branching peaks at 3 years of age, but, by the age of 15–16 years, half of these synapses are lost. Nonhuman primates also exhibit the pattern of synaptogenesis; however, a similar pattern is not seen in rats.

Blood–Brain Barrier Development

Ontogeny of a competent blood–brain barrier (BBB) has been an open discussion since the 1880s when Ehrlich and Goldman identified this restricted space and the term BBB was first coined by Lewandowsky in 1890.⁹² The BBB serves an important function in the development and protection of the mammalian brain. The BBB consists of multiple cell types, including highly specialized endothelial cells, a large number of pericytes embedded in the basal membrane, astrocytic end feet, and perivascular macrophages.⁹³ The formation of tight junction–associated transmembrane proteins, occluding and claudin-5, both involved in BBB function.⁹⁴ These cell types along with the formation of tight junctions, particular patterns of enzymatic activity, a distinct electrochemical gradient, and specific BBB transporters restrict access to the brain for many molecules, for example, glucose and insulin (Figure 11.8).^{92,95–97}

There can be species-specific differences in the amount of a particular drug that reaches and concentrates within the brain during development. This can be related to species differences in postnatal maturation of the BBB.⁹⁸ BBB impermeability occurs at different times at various substances, such that conclusions made about BBB development are often dependent upon both the species studied and experimental technique employed by investigators. The development of the BBB in the human is a gradual process that begins *in utero* and acquires capabilities similar to that of an adult at ~6 months of age.⁹⁹ The development of the BBB occurs during gestation beginning shortly after intraneural neovascularization.¹⁰⁰ The reader is referred to two brief reviews of the research describing the BBB.^{82,93}

When designing a juvenile toxicity study and selecting the age of test species as representative of specific human pediatric populations, the ontogeny of the BBB, both in human and in toxicology test species, can be an important consideration. To properly assess potential CNS toxic compounds, the study must use animals or species of the proper age that provides a comparable level of BBB development with the pediatric population. The use of animals at an age where BBB development is more or less established than the human pediatric population's BBB could under- or overestimate risk.

Watson et al.⁸² concluded that “the rat appears to be an appropriate model for evaluating the risk of toxic effects on postnatal brain development as most of the brain regions/structures that develop postnatally in humans also develop postnatally in rats. However, even though the rat is an appropriate model for most end points, in certain special circumstances other species may be useful for targeted evaluations. There are clear needs for further research, as illustrated by the lack of information regarding development of the BBB, a vitally important structure for understanding the susceptibility of the CNS to toxicity during postnatal development.”

TABLE 11.6
Functional Neurological Landmarks in Humans and Various Experimental Species

Species	Cortical Dominance Established ^a	Sexual Differentiation of the Brain	Mature Cerebral Metabolism (Aerobic Metabolism)	Adult Patterns of Slow Wave and REM Sleep	Fully Mature Prefrontal Cortex (Synaptogenesis and Myelination Complete)
Human	PND 90	Period of plasticity is believed to end before birth Testosterone surge occurs in male neonates between Months 1 and 3	By 1–10 years	10 years	Late adolescence to early adulthood (17–25 years)
Rat	PND 11	Period of plasticity continues after birth Testosterone surge on GD 18–19, and again a few hours after birth	PND 21	PND 32–34	PND 90
Cat				PND 40	
Dog	PND 10	Testosterone surge in males at ~PND 2			
Rhesus monkey		Testosterone surge in males within 2 weeks postnatal			Adolescence/early adulthood ~4–7 years

Source: Adapted from Watson, R.E. et al., *Birth Defects Res. (Pt. B)*, 77, 471, 2006 and references cited within.

Note: PND, postnatal day.

^a Cortical dominance refers to the “smoothing out” of spinal reflexes as higher cortical centers gain control.

TABLE 11.7
Postnatal Neurogenesis and Synaptogenesis

Species	Postnatal Neurogenesis	Peak of Overall Brain Synaptogenesis ^a	Duration of Synaptogenesis	Pruning of Synapses
Human	20% of neurogenesis in the dentate gyrus occurs after birth; neurogenesis slows with age, but a small amount continues throughout life Neurogenesis in the cerebellum completes at 1 year of age	34 week gestation to 2–3 years	Synaptogenesis continues until ~3.5 years of age; the last structure to undergo synaptogenesis is the prefrontal cortex	Synapses are pruned until ~16 years of age. Adult levels of synapses are ~50%–60% maximum values At age of 15 years, a person has about half the synapses he had as a 2 year old
Rat	85% of neurogenesis in the dentate gyrus occurs after birth Neurogenesis in the olfactory bulb continues throughout life	GD 12 to PND 16	Synaptogenesis continues for the first 3 weeks postnatally, peaking in the first 2 weeks	
Rhesus monkey	20% of neurogenesis in the dentate gyrus occurs after birth; neurogenesis slows with age, but a small amount continues throughout life Neurogenesis in the cerebellum completes at 2–3 months of age	GD 40 to PND 61	Synaptogenesis continues for the first two postnatal months, and slowly tapers off	The number of synapses increases during early childhood, plateaus until puberty, then decreases to adult levels due to pruning. The peak number of synapses is reached prior to puberty. At this point, there are ~40% more synapses than in the adult

Source: Adapted from Watson, R.E. et al., *Birth Defects Res. (Pt. B)*, 77, 471, 2006 and references cited within.

Note: PND, postnatal day.

^a This measure is not region-specific.

TABLE 11.8
Age of Onset of Myelination in Various Brain Regions in the Human and the Rat

Brain Region	Human Age (Months)	Rat Age (Postnatal Days)
Cerebellar peduncles	GM 7	7
Lateral lemniscus	GM 6	9
Medial lemniscus	GM 6	10
Brachium, superior colliculus	GM 8	10
Internal capsule	1	10
Stria medullaris	4	10
Habenula peduncular	GM 6	12
Brachium, inferior colliculus	GM 7	12
Fornix	6	12
Corpus callosum and corticospinal tract	1	14
Olfactory tract	2	14
Anterior commissure	3	17

Source: Adapted from Watson, R.E. et al., *Birth Defects Res. (Pt. B)*, 77, 471, 2006 and references cited within.

Note: GM, gestational month.

TABLE 11.9
Times of Acquiring Various Stages of Myelination in the Human, Rat, and Rhesus Monkey

Species	50% Myelination of Corpus Callosum	Myelination Complete
Human	18 months	20–30 years of age
Rat	PND 25	Majority of myelination completes at PND 90–100
Rhesus monkey		Myelination completes by 13 years of age

Source: Adapted from Watson, R.E. et al., *Birth Defects Res. (Pt. B)*, 77, 471, 2006 and references cited within.

Note: PND, postnatal day.

TABLE 11.10
Times of Acquiring Various Growth Characteristics of
Cerebellum in Various Species

Species	Presence of External Granular Layer	50% Adult Weight
Human	Present for at least 1 year, then regresses	1 year
Rat	Becomes prominent shortly before birth; growth in thickness until about PND 10, disappears by PND 21	15 days
Dog	Dwindles from the time of birth until about PND 70	
Rhesus monkey	Disappears at 2–3 months	At birth

Source: Adapted from Watson, R.E. et al., *Birth Defects Res. (Pt. B)*, 77, 471, 2006 and references cited within.

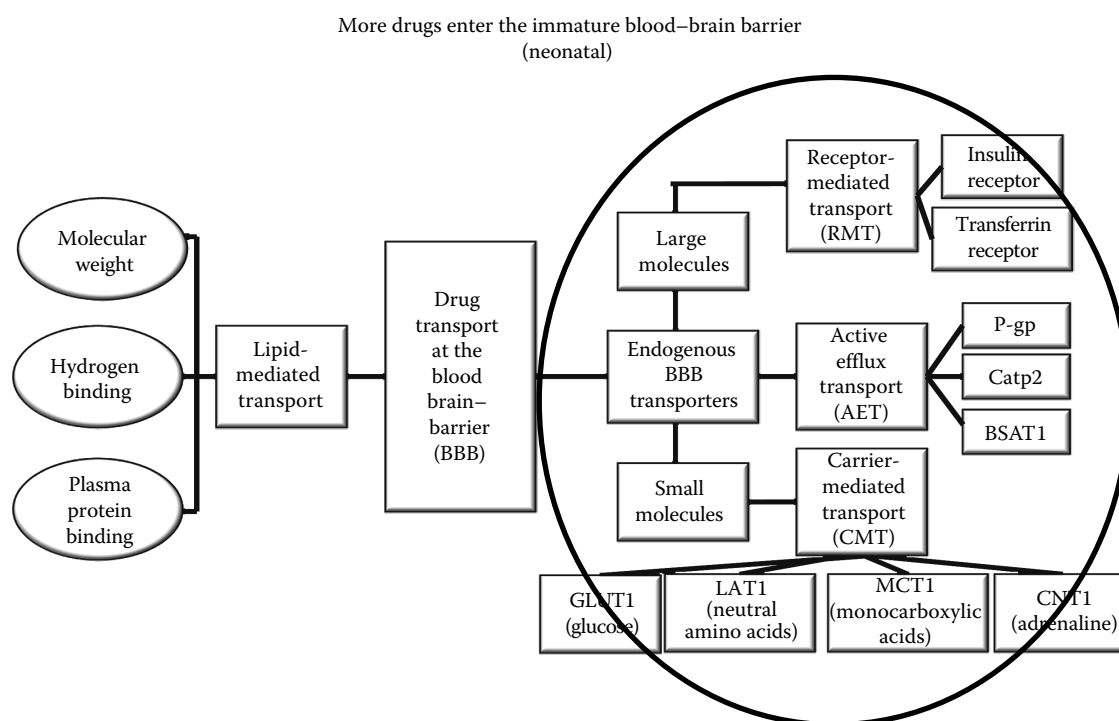


FIGURE 11.8 Outline of a program for developing BBB drug-targeting strategies derived from either chemistry-based or biology-based disciplines. Chemistry-based strategies emphasize lipid solubility, hydrogen bonding, and molecular weight of the drug. Biology-based strategies emphasize endogenous BBB transporters. Small molecules can be transported across the BBB by either accessing certain carrier-mediated transport (CMT) systems or by inhibiting certain active efflux transporters (AET). Large-molecule drugs such as recombinant proteins or gene medicines can be delivered across the BBB via the receptor-mediated transport (RMT) systems. (Reprinted with permission from Yokoyama, H. et al., *Brain Dev.*, 19, 403, 1997; Adapted from Pardridge, W.M., *Drug Discov. Today*, 6, 381, 2001 as reprinted in Pardridge, W.M., *Mol. Interventions*, 3, 2, 2003.)

Neurobehavior

In a review article entitled "Species Comparison of Postnatal CNS Development: Functional Measures," Wood et al.¹⁰¹ identify key events in human postnatal CNS development and compares them, when possible, to other species. Specifically, their review focuses on the behavioral measures of CNS development.

Few would argue with the premise that the ultimate manifestation of a neural process is behavior. The links between the biological processes occurring during CNS development and the behavioral capacities exhibited, however, are not easily established. Behavior is the aspect of postnatal CNS development that is clinically monitored in humans as well as nonclinically in many laboratory species. The maturation of the CNS has been organized in eight categories: (1) locomotor development; (2) ontogeny of fine motor development and dexterity; (3) sensory and reflex development; (4) cognitive development; (5) communication; (6) social play; (7) development of the fear response; and (8) development of sleep cycles.

Locomotor Development

In humans, rats, dogs, and nonhuman primates, locomotor capabilities develop postnatally (Table 11.11). A comparison of motor develop in all these species is beneficial, however, because humans, rats, and dogs all gradually develop from a rather immature state of locomotion (e.g., crawling) to a mature state of walking. Comparisons of locomotor development among humans, nonhuman primates, rats, and dogs are complicated because these two species never walk upright. In addition, human infants cannot readily swim like rats or dogs. Nonhuman primates also develop locomotor abilities postnatally, but at a faster pace than their human counterparts. In rats, dogs, and humans, there seems to be a more gradual rostrocaudal maturation that leads to the shoulders in a raised position before the pelvis.

Assessments of motor development in juvenile toxicity studies would be beneficial in any of these species. Studies in nonhuman primates need to be carefully designed, however, to accommodate the rapid development of locomotion in this species.

Ontogeny of Fine Motor Development and Dexterity

Fine motor skills and dexterity are generally associated with primates, especially humans. Rats, however, possess dexterity in both their fore- and hindlimbs. Although a reflexive ability to grasp objects may be present early in the postnatal life of many species, fine motor skills and other coordinated motor movements develop over time. Fine motor and dexterity development is generally protracted (Table 11.12). Human neonates do not possess the capacity to perform fine finger movements. This capability might not appear until many months after birth. Precise coordination involved with grasping an object and lifting may not be fully mature until the second decade

of life. In contrast, much cruder grasp functions are present at birth. In humans and nonhuman primates, postnatal development of fine motor activity follows a similar pattern, although primates develop much more rapidly. There is a paucity of data in rats and dogs and therefore the use of this measure in routine studies is not likely to provide any benefit for risk assessment.

Sensory and Reflex Development

The various sensory systems develop in a sequential manner. Across a wide range of species, the functional onset of tactile and olfaction usually precedes that of the auditory system.¹⁰² The functional onset of the auditory system precedes that of the visual system. Conditioned responses to various sensory modalities develop in the same sequence,¹⁰³ that is, conditioned responses usually occur first to olfactory, then to auditory, and finally to visual cues.

The sequence of development of sensory and reflex systems in rats, dogs, and nonhuman primates correlate closely with those of the human infant (Table 11.13). For example, the gustatory system in newborn animals is apparently important for selective ingestion and appetitive reflexes, olfaction, and taste are among the first sensory systems to develop. Behavioral responses in humans and rats suggest the presence at birth of functional olfaction and taste systems,^{104–106} and olfaction may even be functional *in utero*.^{107,108} Although the sequence of development of sensory and reflex systems may be similar, timing of development relative to birth varies for some systems. In general, rats, dogs, and humans tend to develop slowly postnatally and have a clearly defined neonatal period during which they are dependent on the mother for shelter and food. However, nonhuman primates have tactile, auditory, and visual systems more developed at birth than in humans or rats, reflecting the need for rapid postnatal development in nonhuman primates. Within 3 months of birth, the infant macaque develops from a state of complete dependence on its mother to one of relative independence.

Cognitive Development

Rats, dogs, monkeys, and human infants have learning capacities that change during development. The capacity to mediate reflexive behaviors emerged before learned behavioral reactions. Vogt and Rudy¹⁰⁹ suggested that the sequence of behavioral development reflects a sequential caudal to rostral maturation of components. It has been well established that subcortical structures are sufficient to support many instances of Pavlovian conditioning,^{110,111} and the lower centers of a sensory system are sufficient to support primitive reflexive behaviors. Higher centers of learning (e.g., prefrontal cortex and hippocampus) are necessary for the acquisition of more complex behaviors. The time required to learn a particular task is often greater in neonates and infants compared to adults. In addition, the accuracy of learning that task is often not equal to an adult. This implies that even though a task can be completed at a fairly young age, it is by no means functionally mature, that is, at adult levels of performance. Finally, it is important to

remember that learning and memory are intimately associated with the maturation of many of the sensory systems.

In all common laboratory species, therefore, evaluations of cognitive function during juvenile toxicity studies can provide insight into the ontogeny of the maturation of these systems (Table 11.14).

Communication

Early in postnatal development, many species use some form of communication to express fundamental needs (e.g., hunger and fear). The complexity of the communication, however, often undergoes a period of prolonged postnatal development. Assessments of communication development in juvenile toxicity studies with rats and dogs (and to some degree in nonhuman primates) are not likely to provide significant insight because communication in these species seems to function primarily as a means of expressing distress (Table 11.15). However in humans, the development of speech and language is a critical component in CNS maturation.

Social Play

Social play is one of the means by which an individual obtains information about his social environment. By playing with peers, the individual learns norms of social communication, social dominance, and social integration in a context where errors in performance usually have neither aversive nor lethal consequences. Evaluations of the social play development in juvenile toxicity studies may provide some insight because it seems to develop postnatally in all common laboratory species (Table 11.16). However, these evaluations would not be straightforward in routine screening studies because methodologies across species are not easily comparable.

Development of the Fear Response

The development of a fear response to a novel environment or stranger is found in many species, including rats, dogs, rhesus monkeys, and human infants. Assessments of the fear response development in juvenile toxicity studies may provide some insight because it seems to develop postnatally in all common laboratory species (Table 11.17). However, methods to evaluate the development of the fear response are not widely applicable to screening toxicity studies.

Development of Sleep Cycles

The sleep–wake cycle is developed postnatally in many species and is thought to be critical in the development of cognitive abilities. Although utilizing sleep–wake cycles as a measure in juvenile toxicity studies will provide limited insight, its development is not well understood (Table 11.18).

The reader is encouraged to refer to the cited text for the specific details of a behavioral assessment and a chapter by Adams¹¹² for a general description of the methods most often utilized in behavioral evaluations.

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was

considered current at the time this guidance was developed (Table 11.19). This comparison should be considered with new information as it becomes available in deciding how best to design appropriate juvenile animal studies to address risks to the pediatric population. Neither the human nor the animal data represent a precise determination of the timelines of development due to the inherent variability and different end points examined. Because of the nature of science, these tables should only serve as a general starting point.

TABLE 11.11
Summary of Locomotor Development

Ability	Human	Rat	Dog	Nonhuman Primate (Rhesus)
Crawling	PND 270 (~9 months)	PND 3–12	PND 4–20	PND 4–49
Walking	PND 396 (~13 months)	PND 12–16	PND 20–28	PND 49 ^a

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

Note: PND, postnatal day.

^a Bipedal locomotion.

TABLE 11.12
Summary of Fine Motor Development and Dexterity

Fine Motor Development	Approximate Postnatal Age Ability Emerges
Human	
Palmar grasping reflex	<2 months
Prehension	5–9 months
Precision grip	1.5–13 years
Rats	
Fine motor development	PND 2–32
Dog	
No information found	No information found
Nonhuman primate	
Fine motor development	11 weeks
Precision grip	3–6 months
Manipulations	6 months

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

Note: PND, postnatal day.

TABLE 11.13**Postnatal Sensory and Reflexive Development of Rat, Dog, Nonhuman Primate, and Human**

Activity	Human	Rat	Dog	Nonhuman Primate
Eating/rooting	Birth	Birth	Birth	Birth
Surface righting	No information found	PND 1–11	No information found	Birth
Grasping/clasping	Birth to 2 months	No information found	No information found	Birth
Orientation	No information found	PND 9–11	No information found	3 weeks
Air righting	No information found	PND 15–18	No information found	5 weeks
Olfaction	No information found	Birth	Birth	No information found
Taste	Birth	Birth	Birth	Birth
	Discrimination	Discrimination	Discrimination	
	Birth to 4 years	Birth to PND 21	Birth to PND 14	
Tactile	Birth	Vibrissa placing: PND 12.5 Nociceptive: PND 21 Tail flick: PND 10–25	Proprioceptive: PND 1 Air puff: PND 10 Nociceptive: PND 1–18 Adultlike nociceptive response: PND 21	Pinprick: birth Mature: 10 weeks
Auditory	Birth	ASR: PND 12 Inhibition of ASR: PND 21–42	ASR: PND 19–25	Birth ASR: PND 14–25
Visual	Visual tracking: birth	Phototaxis response: PND 5	Visual orientation: PND 21–25	Visual tracking: birth
	Perception: mature in 2 months	Eyes open: PND 14–16		Visual orientation: PND 13
	Acuity: mature in 4 to 6 months	Visual placing: PND 17		Acuity: improves during first 2 months of life; mature: 1 year

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

Notes: Approximate age at demonstration of characteristic. Mature, age when animal demonstrates an adultlike response. ASR, Auditory startle response; PND, postnatal day.

TABLE 11.14**Summary of Cognitive Development**

Learning Task	Approximate Postnatal Age Ability Emerges
Humans	
Classical conditioning (air puff)	20–30 days old
Operant conditioning (task-dependent)	2–18 months
Long-term recall	≥13–20 months
Implicit learning	<5 years
Explicit learning	5–9 years
Rats	
Appetitive learning	PND 1
Associative learning (olfactory)	PND 2–8
Active avoidance	PND 9–12
Passive avoidance	PND 10–21
Sequential learning (Biel maze)	PND 17–23
Proximal cued learning (Morris water maze)	PND 17
Spatial learning (Morris water maze)	PND 35–40
Dogs	
Associative learning (olfactory)	PND 1–6
Visual discrimination	5–16 weeks
Delayed response task	≥12–16 weeks

TABLE 11.14 (continued)
Summary of Cognitive Development

Learning Task	Approximate Postnatal Age Ability Emerges
Nonhuman primates	
Conditioned avoidance	PND 3–8
Black–white discrimination task	PND 9–12
Color, pattern, or shape discrimination	PND 20–150
Delayed response task	PND 60 (rudimentary performance) PND 125–135 (improved performance) PND 200–250 (flawless performance)

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

TABLE 11.15
Summary of Communication

Communication	Approximate Postnatal Age Ability Emerges
Human	
“Coos” in response to voices	2 months
Babble with inflection	8 months
Imitates sound	9–10 months
Can understand or say 2 or 3 words with meaning	12 months
Begins to join words together	21–24 months
Rats	
Ultrasonic vocalizations	PND 7–9
USV (maximum)	PND 13
Dog	
Distress vocalizations	3–3.5 weeks
Whining vocalizations	4–5 weeks
Nonhuman primate	
Emotional vocalizations (1 or 2 syllables)	0–6 months
Emotional vocalizations (3, 4, or 5 syllables)	6 months–1 year

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

TABLE 11.16
Summary of Social Play

Social Play	Approximate Postnatal Age Ability Begins to Emerge
Human	40 weeks to 1 year
Rat	PND 14–28
Dog	3–5 weeks
Nonhuman primate	5–9 weeks

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

TABLE 11.17
Summary of Fear Response

Fear Response	Approximate Postnatal Age Ability Begins to Emerge
Human	7–9 months
Rat	PND 20–30
Dog	3–7 weeks
Nonhuman primate	2.5–4 months

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

TABLE 11.18
Summary of Sleep–Wake Cycle

“Semi-Adultlike” Sleep Cycle	Approximate Postnatal Age Ability Begins to Emerge
Human	3–15 months
Rat	PND 15–23
Dog	2–4 weeks
Nonhuman primate	2–8 days

Source: Adapted from Wood, S.L. et al., *Birth Defects Res (Pt. B)*, 68, 391, 2003.

TABLE 11.19
Species Comparison of the Development of the Central Nervous System

Developmental Event	Postnatal Development Period			
	Human (Years)	Primate (Weeks)	Dog (Weeks)	Rat (Days)
Glutamate receptors ^a (maximal binding)	1–2 Cortex decline to adult 2–16			28 Decline to adult >28
Monoamine system ^b	2–4 Maximum receptor density			21–30 Adult levels
Ocular dominance ^c	0–3			21–35
Cerebellum persistent external granular layer ^c	0.6–2			0–21
Rapid phase of myelination ends ^d	2			25–30
Cognitive development delayed response learning ^e	1–2	9–36	12–16	10–35

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

^a Ikonomidou et al.¹⁵

^b Rice and Barone.²¹

^c Sidhu et al.¹⁶ and Kimmel and Buelke-Sam.¹⁵⁸

^d Radde.²²

^e Wood et al.¹⁰¹

CARDIOVASCULAR

In a review article on comparative heart and vascular development, Hew and Keller¹¹⁴ discuss the critical events in heart postnatal development, growth and maturation of the human heart and its vascular bed, and explain how the infant/juvenile systems differ, both morphologically and functionally, from that seen in the adult. The postnatal changes observed in the heart are relatively similar across the mammalian species,

but do differ in the timing of events (Table 11.20). As a general rule, the functional and morphological changes in the heart occur faster, according to the developmental rate, in small laboratory animals compared to humans due to the faster growth and maturation rate in these animals.

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Table 11.21).

TABLE 11.20
Species Comparison: Summary of Postnatal Heart Development

Parameter	Human	Rat	Dog
Heart size/shape	Heart position higher and more transverse; oblique lie attained between 2 and 6 years of age. At birth, ventricular volumes are equal. Right ventricular volume doubles by 12 months, left ventricular volume unchanged. R/L ratio reaches 2:1 at 2 years	Age-related changes in ventricular dimensions. Becomes more spherical with age	Age-related changes in ventricular dimension. Becomes more spherical with age
Heart weight	Relative heart weight constant Absolute weight doubled by 6 months and tripled by 1 year; achieved adult relative to body weight by about 21 years	Decreasing relative heart weight with age High weight increases on PND 1–5 with slower growth afterward	Decreasing relative heart weight with age
Cardiac cells	Myocyte count at birth is 50% in adult, with proliferation, adult value reached by 4 months. Growth thereafter due to myocyte hypertrophy: 5 mm at birth, 8 mm at 6 weeks, 11 mm at 3 years, 13 mm at 15 years, and 14 mm in adults Myocytes diploid at birth, compared to 60% diploid, 40% polyploid in adults	Myocyte proliferation (three- to fourfold increase) and hypertrophy from birth to 2 months of age. Myocyte diameter 5.5 mm at 14 days, 10.5–11.8 mm at 30 days and 15–16 mm in adults Myocytes primarily diploid in both infant and adult	Myocardial cell numbers relatively constant. Growth primarily through myocyte hypertrophy: 7 mm at 100 days, 13 mm at 0.5–0.9 year and 14 mm at 1–4.2 years. No information available
Coronary vasculature	Primary arteries established before birth, diameter of coronary arteries doubled at first year reaching maximum at 30 years of age Some capillary angiogenesis occurs postnatally. Capillary density decreases with age	More immature at birth. Capillary and arteriole angiogenesis occurs postnatally. Arterial maturation by 1 month Volume fraction of capillaries reaches maximum of 16% by Day 28. Capillary density subsequently decreases with age	Minimal information available. Capillary angiogenesis occurs postnatally
Cardiac innervation	Morphologically and functionally immature at birth. Numbers of neurons gradually increase and reach maximum density and adult patterns in childhood	Morphologically and functionally immature at birth. Adrenergic patterns complete by 3 weeks and nerve density completes by 5 weeks. Cholinergic and other nerve types also matured postnatally	Morphologically and functionally immature at birth and continues development during the first 2–4 months of age
Cardiac output and hemodynamics	Decreasing basal heart rate: 138 beats/min at birth to 55–85 beats/min in adults Infants and young children have smaller ventricular volumes, stroke index, and ejection fractions, but no differences in cardiac index compared to adults Rapid increase in blood pressure from birth to 2 months (systolic/diastolic, 62/40 to 85/47); relatively constant from 6 months to 8 years of age (diastolic 58–62)	Systolic blood pressure doubles from neonate to young adults and reaches adult levels by 10 weeks of age	No information available

Source: Adapted from Hew, K.W. and Keller, K.A., *Birth Defects Res. (Pt. B)*, 68, 309, 2003.

TABLE 11.21
Postnatal Development Period (Maturation Level Similar to Adult)

Cardiac Parameter	Postnatal Development Period (Maturation Level Similar to Adult)		
	Human (Years)	Dog	Rat (Weeks)
Electrophysiology (ECG)	5–7	NA	3–8
Cardiac output and hemodynamics	Birth 138 beats/min Adult 85 beats/min <2 years smaller ventricular volume, stroke index, and ejection fraction versus adult Blood pressure: Birth—62/40 2 months—85/47 0.5–8 years—Diastolic 58/62	Increase in blood pressure and decrease in heart rate from 1 week to 6 months	Early increase in heart rate then constant into adulthood High cardiac output and low PVR Neonate–puberty systolic blood pressure doubles; reaches maturity by 19 weeks
Myocytes	Diploid at birth (compared 60% in adults (40% polyploidy)	NA	Primarily diploid in infants and adults
Coronary vasculature	Diameter of arteries doubled at 1 year; maximum at 30 years; capillary angiogenesis occurs postnatally and density decreases with age	Capillary angiogenesis occurs postnatally and density decreases with age	Capillary angiogenesis occurs postnatally; arterial maturation by 1 month
Cardiac innervation	Neuron number increase and reach adult pattern/density in childhood	Continued development during 2–4 months	Adrenergic pattern matures by 3 weeks and nerve density matures by 5 weeks Cholinergic matures postnatally

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

GASTROINTESTINAL

In a review article on comparative GI system development, Walthall et al.²⁶ summarizes available literature regarding the postnatal development of the GI system. The organs of the GI tract are generally well developed at birth, but some structural and considerable functional development occurs postnatally. There is more species variability for functional maturation, but the functional development pattern is relatively consistent between species. The major driver for the functional changes is alteration in diet. But because the developmental patterns are similar for laboratory animals and humans, postnatal GI development can be assessed in appropriately designed juvenile toxicity studies. No single laboratory species appears to be a superior model for human postnatal GI development. However, differences in postnatal GI maturation exist between species that could be important factors when interpreting studies conducted in the developing human or animal.

The scientific literature contains reports of postnatal GI development being dependent upon several factors including

diet, age, genetic determinants, hormones secreted from the intestine, hormones secreted from other portions of the body, and various other factors (e.g., growth factors). In mammals, at birth there is an abrupt change in diet from amniotic fluid to milk. At weaning, a change occurs again as milk is gradually substituted with solid food as the primary source of nutrition, thus transitioning from a high-fat, low-carbohydrate diet to a low-fat, high-carbohydrate diet.^{115,116} The postnatal development of the GI tract has been divided into the three periods: (1) the birth and early suckling period, (2) the suckling period, and (3) the weaning period.¹¹⁷ The weaning period also marks the beginning of notable changes in digestive and transport function that results in maturation of the GI system.

Stomach

Postnatal development of the human stomach involves thickening of the glandular region and the maturation of chief cells. During the first months postpartum, the number of chief cells increases, gradually increasing the volume of

hydrochloric acid and pepsin secretions. Bacterial colonization occurs with this functional maturation. The postnatal development pattern similar to that of humans was observed in the stomach of the guinea pig, pig, and rabbit; however, species variation exists with regard to the timing and sequence of appearance of specialized glandular cells. At birth, hydrochloric acid secretion does not occur in the human, rat, or dog stomach; however, both the rabbit and pig stomach secrete hydrochloric acid.

Intestine

The degree of intestinal maturation at birth varies among species. In general, the maturity of the intestine at birth is considered to be dependent upon the length of the gestational period. Species such as the mouse and rat have a short gestational period and are thus very immature at birth. Other species such as the guinea pig and pig have longer gestational periods and have more developed intestines at birth. In animals that have relatively long gestational periods, intestinal development during the gestational and early postnatal period is more similar to that of humans than intestinal development in animals with short gestational periods (Table 11.22).

- *Small intestines (SI)*: In humans, the crypts and villi become organized and functional well before birth and the primary postnatal changes are growth and functional changes in the intestinal mucosa, bacterial colonization, and intestinal motor and peristaltic activity. The SI in the rat is structurally and functionally immature at birth. Postnatal maturation involves a change in the cell population covering the villi and replacement of the immature epithelium with the mature epithelium. The SI of rabbits is structurally and functionally immature at birth but matures within about 2 months. The SI of the pig exhibits changes in both structure and function; however, most adult characteristics are attained by postnatal week 3. The GI tract is fully formed in dogs at birth, but as with humans, postnatal functional development occurs as the dog matures.
- *Large intestines (LI)*: In the human LI, intestinal crypts develop during the late gestational period and by birth the proliferative activity in the crypts is generally organized like the adult. Enzymatic activity develops in the human LI before birth but sodium absorption and anion exchange, important for maintaining electrolyte balance, are not well developed at birth but develop rapidly during the first year of life.

The rat LI is morphologically and functionally immature at birth; however, during the postnatal period the lumen of

the LI enlarges, intestinal crypt lengthening occurs, and the number of mucosal cells increases such that by the time of weaning (PND 21) a mature adult morphological appearance is established. Enzyme activity is present in the rat LI before birth, and adult levels are achieved within the first 2–3 weeks of postnatal development.

The rabbit LI is also immature at birth, but the mucosa matures markedly, reaching maturity by 2 months postpartum. The pig LI changes significantly during the first few PND, achieving an adultlike appearance by PND 6.

Liver

As an accessory organ of digestion, the liver functions as an exocrine gland to secrete bile. The human liver achieves an adultlike appearance by 5 years of age. Structural changes occurring during postnatal maturation of the human liver include thinning of the hepatic plates as well as increases in the size of liver cells, hepatic lobule diameter, and amount of endoplasmic reticulum. During the neonatal period, bile flow is substantially less than adult levels and bile acids are mostly conjugated with taurine, but the glycine/taurine biliary salt composition resembles that of adults by 7–12 months.

Postnatally the rat liver undergoes structural changes similar to those seen in human, and at 1 month postpartum maturation is essentially complete. Hepatic bile flow in the rat decreases with age during the first postnatal year.

Changes in the number and size of hepatocytes in the rabbit occur during postnatal development. Bile flow and the secretion of bile salts are low immediately after birth but progressively increase to levels approaching that of adult animals by weaning.

In the dog, most structural maturation occurs during the first 3 days after birth, with full maturity of the biliary tree occurring by the end of first postnatal week. Endogenous production of bile acids, mean bile output, and bile flow is low at birth and increases with age, but the hepatic mechanisms of bile secretion and excretion seem to be fully developed at birth. By 4 weeks of age, bile acid concentration was similar to that of adult dogs (Table 11.23).

Pancreas

The exocrine pancreas secretes enzymes that aid in the digestion of lipids, proteins, and carbohydrates and the endocrine pancreas secretes substances that aid in the neutralization of the stomach contents as they enter the SI. At birth, the morphology of the human pancreas is similar to that of the mature organ but the pancreas is not functionally mature until around 2 years of age. As with humans, the pancreas of most mammalian animals is morphologically but not functionally mature at birth. In general, functional maturity of the pancreas in animals occurs around weaning (Tables 11.24 and 11.25).

TABLE 11.22
Species Comparison of the Development of the Gastrointestinal System

Parameter	Rat	Rabbit	Pigs	Dogs	Human
General	The maturity of the intestine at birth is thought to be dependent upon the length of the gestational period				
Stomach	Divided into a glandular region with columnar secreting epithelium and a nonglandular region lined with stratified squamous epithelium	Not sectioned and has thin walls	Possess a glandular stomach that contains cardiac, gastric, and pyloric mucosa. The gastric mucosa covers a larger portion of the stomach compared to humans	Possess a glandular stomach that contains cardiac, gastric, and pyloric mucosa	Possess a glandular stomach that contains cardiac, gastric, and pyloric mucosa
Small intestine (SI)	At birth, the SI is immature with postnatal mucosal maturation occurring during the first 3 postnatal weeks (PNW). Crypts and villi lengthen with an increase in cell numbers and mature mucosal cell numbers. The wall thickness doubles due to the change in diet that occurs with weaning (PND 17–26)	At birth, the SI is structurally and functionally immature. The duodenal glands and the muscularis are absent. Duodenal glands appeared during PND 1–2 and the muscularis on PND 5. SI is morphologically mature by PND 20, but not developmentally mature until PNW 8	SI exhibits changes in both structure and function through PNW 3, when adult characteristics are attained At weaning (PNW 2, 3, or 4), the developmental activity of crypt changes with the depth lengthened and villus length shortened	GI tract is fully formed at birth but postnatal functional development occurs. The most rapid period of growth is seen from birth to PND 63 when postnatal mucosal growth (lengthening of the villi and crypts) occurs. From PND 63 to adulthood, no changes in crypt or villus dimensions occur	Intestinal crypts are generally formed during gestation. Intestinal motor activity is less frequent and the peristaltic activity is irregular during infancy. By late infancy, most GI functions similar to adults. The SI grows continually with the surface area increasing by >40-fold at adulthood
Large intestine (LI)	The LI is immature at birth, the colonic crypts are ~½ adult lengths with cell population substantially less than adult. As the colon matures, the number/length of crypts increase, primary and secondary folds form, and the number of cells increase. By weaning (PND 22–26), adult morphological appearance is established	The thickness of the colonic muscularis increases from PNW 2 to 4, decreases by PNW 8, and exhibits a marginal increase by PNW 16. Villus-like structures are evident in the cecum and colon during PNW 2, but by PNW 4, the villus-like structures begins to disappear coinciding with weaning (~PNW 4). By PNW 8, the depth of the mucosa and thickness of the muscularis has increased, but no further increases are seen. The thickness of the cecum muscularis decreases from PNW 2 to 16	At birth, villus-like epithelium projections in the colon has the ability to absorb lipids and actively transport amino acids similar to SI villi. Lining the villus-like structures are goblet, absorptive, and vacuolated cells. During PND 1–3, the villus-like structures, vacuolated cells, and nutrient absorption are lost and the adult characteristics are attained. By PND 6, the epithelial surface is flat, contains crypts and goblet cells, but is devoid of villi		Crypts develop during the late gestation. The vacuolated columnar cells, mucous cells, and endocrine cells of the crypts are present at birth. After birth, a 100-fold increase in the number of intestinal crypts occurs with a marked increase in colonic mucosal size. Unlike in the SI, villi which are present during gestation are not seen in the LI at birth

Source: Developed from Walthall, K. et al., *Birth Defects Res. (Pt. B)*, 74, 132, 2005 and references cited within.

TABLE 11.23
Species Comparison of the Development of the Liver

Rat	Rabbit	Pigs	Dogs	Human
<p>At PND 8–28, the percent of single plates increased dramatically and the maturation of the liver included extensive remodeling of the liver structure. At PND 28, the maturation of the liver was essentially complete and similar to that seen in the mature human liver. The liver weight increased significantly from PNW 4 to 9. The increases in liver growth were parallel to linear increases in body weight. The liver weight increased during the first year and then the liver weight did not increase further. The liver to body weight ratio was greatest at 1 month, and by 3 months of age it was similar to that of rats aged 12 and 24 months.</p>	<p>During PNW1, <5% of the cells in the liver were not hepatocytes, accounting for <1% of the total volume of the liver. The size of hematopoietic cells increased slightly after PNW 1 through PNW 8. Hepatocyte size decreased during PNW 1, after which a threefold increase to adult hepatocyte size occurred. Over the first 24 h after birth, the liver did not significantly change in weight. But by PND 7, the weight liver in suckled rabbits was approximately twice that seen at birth. Liver growth in rabbits occurs as a result of hyperplasia, similar to that seen in rats after birth.</p>	<p>The liver did not grow significantly during PND 1. From PND 2 to 9, the liver weight increased by about 9.4 g/day. By PND 10, the liver weight had increased by about threefold.</p>	<p>Most structural maturation of liver cells (hepatocytes and bile ductular cells), and the biliary tree and associated passages occurs by PND 3, with full maturity of the biliary tree occurring by the end of PNW 1. In newborn dogs, the liver to body weight ratio is approximately twofold greater than adult. When comparing bile secretory function in young dogs with that of adult, the liver weight must be used as the basis for comparison and not the body weight.</p>	<p>At birth, the hepatocytes that make up the hepatic cords or plates are usually arranged such that the plates are at least three cells thick. As the liver matures, the morphology changes such that the hepatic plates thin to the characteristic one cell-thick appearance seen in adults. By PNW 21–25, multilayered plates are still dominant in the liver. The additional layers in the hepatic plates of the infant function to control the entry and exit of substances from the sinusoids. By 5 years of age, the liver attains full maturity, characterized by hepatic plates that are one cell thick.</p>

Source: Developed from Walthall, K. et al., *Birth Defects Res. (Pt. B)*, 74, 132, 2005 and references cited within.

TABLE 11.24
Species Comparison of the Development of the Pancreas

Rat	Pigs	Human
<p>At birth, the pancreas is essentially morphologically mature. The functional development occurs at the time of weaning (PND 21). During the PND 1–3, pancreas weight decreased by 48% relative to body weight (33% absolute weight decrease). It was suggested that the decrease in pancreas weight during this time period was due to the release of accumulated pancreatic enzymes in response to suckling. After the initiation of weaning, rapid increases in pancreas weight have been observed. A few days after the commencement of weaning (which began on PND 14 and ended on PND 21), rapid increases in pancreas weight occurred. The most rapid increases in pancreas weight were seen between PND 17 and 20.</p>	<p>The postnatal maturation of the exocrine pancreas is more dependent upon weaning than upon age. During postnatal development, the pancreas weight increased substantially before weaning, which occurred at PND 28. During PND 1–3, the pancreas grew quite quickly, almost doubling during the first 24 h of life. The weight of the pancreas increased 70% relative to body weight (97% absolute weight increase). A 50% increase in mean pancreatic cell size occurs in the first 24 h after birth, as evidenced by the significant increase in protein but not DNA. By PND 10, the amount of DNA increased to twice that seen at birth. After weaning (PND 28), substantial increases in pancreas weight occurred.</p>	<p>In human neonates, the morphology of the pancreas is similar to that of the mature organ; however, the pancreas is not functionally mature at birth. Although the pancreas of newborns is similar to that of the adult, some aspects of structural development (i.e., proliferation of pancreatic epithelial cells to increase pancreatic tissue) extend into early childhood. Functional development of the pancreas extends further than does morphological development. The human pancreas is not functionally mature until around the age of 2 years.</p>

Source: Developed from Walthall, K. et al., *Birth Defects Res. (Pt. B)*, 74, 132, 2005 and references cited within.

TABLE 11.25

Species Comparison of the Development of the Pancreatic Enzymes

Rat	Pigs	Human
<p>During PND 1–3, the activity of pancreatic enzymes such as amylase, trypsin, chymotrypsin, and lipase decreased in the rat pancreas. Digestive enzymes of the pancreas decrease notably in pups once suckling begins. Functional maturity is partially dependent upon weaning and hormonal secretions. The change in diet at weaning is accompanied by a prompt increase in hydrolases (primarily amylase) in the pancreas. If nursing is prolonged, then the normal increase in pancreatic amylase is delayed and if rats were weaned to a high-fat, low-carbohydrate diet, a reduced amount of pancreatic amylase secretion occurred. Postnatal changes in the hormone levels (such as glucocorticoids and thyroxine) affect the functional development of the pancreas. Hormones (e.g., secretin, cholecystokinin [CCK], gastrin, bombesin, epidermal growth factor [EGF], insulin, and pituitary hormones) may also modulate postnatal pancreatic enzymatic production.</p>	<p>The secretion of proteolytic enzymes in the pancreas increases from PND 4 to 36. The levels of trypsin, chymotrypsin, and amylase present at birth are less than adults; however, the levels of these enzymes are substantial enough to induce hydrolysis of proteins. By PND 3, pigs were able to secrete pancreatic juice in response to exogenous and endogenous secretion. During postnatal development, the pancreatic activity of trypsin, chymotrypsin, and amylase increases. Some pancreatic enzymes have increased activity at PNW 1 such as amylase (336%), while trypsin, chymotrypsin, and lipase activity did not significantly change. Amylase activity increases quickly up to PNW 4–5. Other enzymes have increased activity at weaning (PNW 3–4). The maturation of pancreatic exocrine function is more dependent upon weaning than upon age. Trypsin, chymotrypsin, and amylase activity have been shown to increase before weaning, transiently decrease after weaning, and subsequently increase further. A transient decrease in trypsin, chymotrypsin, and amylase activity occurs after weaning (PND 28), but increases in enzyme activity occurred thereafter. The secretion of pancreatic lipase is elevated at birth, increases during suckling, and decreases after weaning.</p>	<p>At birth, the pancreas does not have full secretory capacity and does not respond to hormonal stimuli. During the first postnatal month, the pancreas does not secrete enzymes in response to cholecystokinin or secretin. Pancreatic lipase levels are significantly less at birth (one-fourth adult level), while trypsinogen levels are slightly less. Amylase levels are virtually nonexistent for the first 3–4 postnatal months and notably increasing thereafter. Lipase activity has been shown to begin to increase rapidly during PNW 10. Trypsin and lipase secretion exhibit a 10-fold increase by postnatal month 9, while at birth trypsin activity was ~90% of childhood levels, increased during the first 3 postnatal weeks, and did not increase much after this point. The activity of chymotrypsin at birth has been shown to be ~60% of that seen in children and has been shown to increase on PD 4. Carboxypeptidase levels at birth were shown to be one-fourth that seen in children. Enterokinase levels at birth are only 17% of that seen in young children (1–4 years old). As a result of low pancreatic lipase levels and low bile acid concentration in newborns, fat digestion can be poor in human infants.</p>

Source: Developed from Walthall, K. et al., *Birth Defects Res. (Pt. B)*, 74, 132, 2005 and references cited within.

CYP450 ENZYMES

The reader is referred to two review articles by de Zwart et al.¹¹⁸ and Hines and McCarver⁵¹ concerning the ontogeny of drug-metabolizing enzymes and transporters in rats and humans. Ontogeny of the transporters depended on the transporter and the organ studied. Changes in mRNA expression of the various transporters during development are likely to result in altered elimination and/or tissue distribution of substrates, with concomitant changes in hepatic metabolism, renal excretion, and passage through the BBB.

The following information is from the review article by Zwart et al.¹¹⁸ Although some human Phase I drug-metabolizing enzyme developmental expression patterns are beginning to emerge, knowledge remains far from complete. Expression of a few enzymes has been observed as early as organogenesis. In the fetal liver, at least two of the enzyme families that are expressed exhibit a temporal switch in the immediate perinatal period (e.g., CYP3A7 to CYP3A4/3A5 and FMO1 to FMO3), whereas others show a progressive change in isoform expression through gestation (e.g., the class I alcohol dehydrogenases). Many of the Phase I drug-metabolizing enzymes exhibit dynamic perinatal expression changes that are

regulated primarily by mechanisms linked to birth and secondarily to maturity. A few of these enzymes are not detectable until well after birth, suggesting that birth is necessary but not sufficient for the onset of expression (e.g., CYP1A2). Tissue-specific expression adds to the complexity during ontogeny. For example, CYP3A7 expression is restricted to the fetal liver. However, with few exceptions, complete temporal relationship information during development is not known. Furthermore, most studies have concentrated on hepatic expression and much less are known about extrahepatic developmental events.

Knowledge of the ontogeny of the various systems involved in distribution and elimination of drugs is important for adequate interpretation of the findings during safety studies in juvenile animals. Consideration of the ontogeny of metabolizing enzymes and transporters may improve the design and interpretation of results of toxicity studies in juvenile animals.

Activity and mRNA expression of various metabolizing enzymes in the rat liver and brain (cerebellum) are shown in Tables 11.26 through 11.28. In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Table 11.29).

TABLE 11.26
mRNA Expression of Various Transporters in the Rat Liver (Relative to mRNA Expression at Day 42)

			Day -1	Day 1	Day 4	Day 7	Day 11	Day 15	Day 18	Day 21	Day 26	Day 42
Parameter			ABC Transporters									
Mdr1a	mRNA	M	↓*	=	↓* ^a	↓*	=	↑*	↑	↑*	↑	—
		F	↓*	↓	↓*	↓*	=	↑*	↑	↑*	↑	—
Mdr1b	mRNA	M	↓*	↑	↓*	↓*	↓	↓	↓	↓	↓	—
		F	↓*	↓	↓*	↓*	↓	↑	↑	↓	↑	(51%) ^a
Mrp1	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	=	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	=	—
Mrp2	mRNA	M	=	=	↓*	↓*	↓*	↓*	↓*	↓*	↓*	—
		F	↓	↓	↓*	↓*	↓*	↓*	↓*	↓*	↓*	(149%) ^b
Mrp3	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	(160%) ^b
Mrp6	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	(62%) ^a
Bsep	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑	↑	↑	↑	—
		F	↑*	↑*	↑*	↑*	↑*	=	↑	↑	↑	—
Bcrp	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	=	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↓	—
			S1c Transporters									
Oct1	mRNA	M	↓*	↓*	=	=	=	↑	↑*	↑	↑	—
		F	↓*	↓*	=	=	↓	↑	↑*	↑	↑	—
Oct2	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑	—
		F	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Oat1	mRNA	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		F	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Oat2	mRNA	M	↓*	↓*	=	=	↑	↑*	=	↑*	↑	—
		F	↓*	↓*	=	=	=	↑*	↑	↑*	↑	—
Oat3	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↓*	↓*	↓*	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	(3%) ^a
Oatp1a4	mRNA	M	↓*	↓*	↓*	↓*	↓*	=	=	↑*	↑	—
		F	↓*	↓*	↓*	↓*	↓*	↑	↑	↑*	↑	(67%) ^a

Source: Adapted from de Zwart, L. et al., *Reprod. Toxicol.*, 26, 220, 2008.

Notes: (↓) Level/activity less than that at Day 42; (=) Level/activity equal to that at Day 42; (↑) Level/activity greater than that at Day 42; (↑) Peak level. NA, Not analyzed; ND, not detectable.

^a Level/activity at Day 42 for females less than that in males (% relative to male level at Day 42).

^b Level/activity at Day 42 for females greater than that in males (% relative to male level at Day 42).

*Significantly different from adult levels ($p < 0.05$).

TABLE 11.27

Activity and mRNA Expression of Various Metabolic Enzymes in Rat Liver Microsomes
(Relative to Activity/mRNA Expression at Day 42)

Parameter			Day -1	Day 1	Day 4	Day 7	Day 11	Day 15	Day 18	Day 21	Day 26	Day 42
CYP1A1/2	Activity	M	↓	↓	↓	↓	↓	↓	=	↑	↑	—
		F	↓	↓	↓	↓	↓	↓	=	↑	↑	—
CYP1A1	mRNA	M	↑	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
		F	↑	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
CYP1A1/2	mRNA	M	↓*	↓*	↓*	↓*	↑	↑*	↓*	↓*	↑	—
		F	↓*	↓*	↓*	↓*	↑	↑*	↓*	↓*	↑	—
CYP2B1/2	Activity	M	↓	↓	=	=	↑	↑	↑	↑	↑	—
		F	↓	↓	=	=	=	↑	↑	=	=	—
CYP2E1	Activity	M	↓	↓	↑	=	↑	↑	↑	↓	↓	—
		F	↓	↓	↑	↑	↑	↑	↑	=	=	(71%) ^a
	mRNA	M	↓*	↓	=	↑	↑*	↑*	↑*	↑	↑	—
		F	↓*	↓	=	↑	↑*	↑*	↑*	↑	↑	—
CYP3A1/2	Activity	M	↓	↓	↓	↓	↓	↓	↓	↓	=	—
		F	↓	↓	↓	↓	↓	↓	=	=	=	—
CYP3A2	mRNA	M	↓	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
		F	↑	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	(2%) ^a
CYP4A1	Activity	M	↓	↓	↓	↑	=	↑	↑	↑	=	—
		F	↓	↓	↓	=	↓	↑	=	=	↓	—
	mRNA	M	=	↑*	=	=	=	↓	↓*	↓	↑*	—
		F	=	↑*	=	=	=	↓	↓*	=	↑*	—
Carbonyl esterase	Activity (liver)	M	↓	↓	↓	↓	=	↓	↓	↓	=	—
		F	↓	↓	↓	↓	↓	↓	↓	↓	↓	—
	Activity (Plasma)	M	↓	↓	↓	↓	↓	↓	↓	↓	=	—
		F	↓	↓	↓	↓	↓	↓	↓	↓	↓	(137%) ^b
T4-GT	Activity	M	↓	↓	↓	↓	↓	=	=	↓	↑	—
		F	↓	↓	=	=	=	=	=	=	↑	(31%) ^a

Source: Adapted from de Zwart, L. et al., *Reprod. Toxicol.*, 26, 220, 2008.

Notes: (↓) Level/activity less than that at Day 42; (=) Level/activity equal to that at Day 42; (↑) Level/activity greater than that at Day 42; (↑) Peak level.
 NA, Not analyzed; ND, not detectable.

^a Level/activity at Day 42 for females less than that in males (% relative to male level at Day 42).

^b Level/activity at Day 42 for females greater than that in males (% relative to male level at Day 42).

*Significantly different from adult levels ($p < 0.05$).

TABLE 11.28
mRNA Expression of Various Transporters in the Rat Cerebellum (Relative to mRNA Expression at Day 42)

Parameter			Day -1	Day 1	Day 4	Day 7	Day 11	Day 15	Day 18	Day 21	Day 26	Day 42
ABC Transporters												
Mdr1a	mRNA	M	=	=	↓	↓*	↓*	=	=	↑	↑	—
		F	=	↑	↓	↓*	↓*	=	=	↑	↑	—
Mdr1b	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	—
Mrp1	mRNA	M	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑	—
		F	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑	(149%) ^a
Mrp2	mRNA	M	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		F	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mrp3	mRNA	M	↑	↑*	↑*	↑	=	=	↓	=	↓	—
		F	↑	↑*	↑*	↑	=	=	↓	=	↓	(195%) ^a
Bcrp	mRNA	M	↑	↑*	=	↓	↓	↑*	↑	↑*	↑*	—
		F	↑	↑*	=	↓	↓	↑*	↑	↑*	↑*	—
S1c Transporters												
Oct1	mRNA	M	↓	↓	=	↓	↓	↓	↓	↓	↓	—
		F	=	=	↑	↓	↓	↓	↓	↓	↓	(63%) ^b
Oct2	mRNA	M	↓	↓	↓	↓	↓*	↓	↓	↓	↓	—
		F	↓	=	↑	↓	↓*	↓	↓	↓	↓	(49%) ^b
Oat1	mRNA	M	↓	↓	↓	↓	↓	↓	↓	↓	↓*	—
		F	↑	↑	↑	=	↓	=	↓	↓	↓*	(40%) ^b
Oat2	mRNA	M	↑	↑*	↑	=	↑	↑	↑	↑	↓	—
		F	↑	↑*	↑	↓	↑	↑	↑	↑	↓	(254%) ^a
Oat3	mRNA	M	↓	↓	↓	↓	↓	↓	↓*	↓	↓	—
		F	↓	↓	↓	↓	↓	↓	↓*	↓	↓	(174%) ^a

Source: Adapted from de Zwart, L. et al., *Reprod. Toxicol.*, 26, 220, 2008.

Notes: (↓) Level/activity less than that at Day 42; (=) Level/activity equal to that at Day 42; (↑) Level/activity greater than that at Day 42; (█) Peak level. NA, Not analyzed; ND, not detectable.

^a Level/activity at Day 42 for females greater than that in males (% relative to male level at Day 42).

^b Level/activity at Day 42 for females less than that in males (% relative to male level at Day 42).

*Significantly different from adult levels ($p < 0.05$).

TABLE 11.29
Developmental Modulation of Phase I/II Metabolism

Developmental Modulation of Phase I/II Metabolism			
Enzyme	Human (Years)	Rat (Days)	Rabbit (Days)
CYP2D6 ^{a,b}	0–3	NA	NA
CYP2E1 ^{b–d}	0–1	4–17 Decreased postweaning male > female	13–35 2 × Adult at 35
CYP1A2 ^{a,e–g}	0.5–1 (>Adult)		21–60
CYP2C8 ^{a,b}	<1	NA	NA
CYP2C9 ^{a,b}	<0.5–9.5 (>Adult)	NA	NA
CYP3A4 ^b	0–2	NA	NA
Acetylation ^{a,b}	1 (35% Adult)	NA	NA
Methylation ^{a,b}	<1 (50% Adult)	NA	NA
Glucuronidation ^{a,b}	0 (>Adult) 12	NA	NA
Sulfatin ^{a,b}	0	NA	NA

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

Note: NA, Not available.

^a Kearns and Reed.¹⁵⁹

^b Leeder and Kerns.¹⁶⁰

^c Waxman et al.¹⁶¹

^d Peng et al.¹⁶²

^e Ding et al.¹⁶³

^f Imaoka et al.¹⁶⁴

^g Pineau et al.¹⁶⁵

IMMUNE SYSTEM

In a review article on comparative immune system development, Holsapple et al.¹¹⁹ compare the anatomical and functional development of the immune system in several species important to either preclinical studies for drug development or safety assessments for chemicals, with what is known in humans (Table 11.30). Unfortunately, the components of the immune system have not been traditionally emphasized as potential target organs in standard developmental and reproductive toxicity (DART) protocols although histopathologic evaluation of the lymphoid organs is now a part of General Toxicity Studies. A key question that needs to be addressed in the development of a juvenile toxicity study is the determination of the most appropriate species and strains to model the developing human immune system.

The immune system develops from pluripotential hematopoietic stem cells (HSC) that are generated early in gestation from uncommitted mesenchymal stem cells in the intraembryonic splanchnopleure surrounding the heart. HSC are a population of multipotential stem cells that retain the capacity to self-renew and that have the capacity to differentiate to form all subclasses of leukocytes that participate in immune responses, as well as megakaryocytic and erythrocytic cells.¹²⁰ These HSC can differentiate via migration

through an orderly series of tissues, and a dynamic process that involves continual differentiation into lineage-restricted stem cells. Establishment of the lymphoid–hematopoietic progenitor cells involves the migration of these cells from intraembryonic mesenchyme to fetal liver and fetal spleen, and ultimately, the relocation of these cells during late gestation to thymus and bone marrow. The latter two organs are the primary sites of lymphopoiesis and seem to be unique in providing the microenvironmental factors necessary for the development of functionally immunocompetent cells. The lineage-restricted stem cells expand to form a pool of highly proliferative progenitor cells that are capable of a continual renewal of short-lived functional immunocompetent cells, and that ultimately provide the necessary cellular capacity for effective immune responsiveness and the necessary breadth of the immune repertoire.¹²¹ It is important to realize that immune system development does not cease at birth, and that immunocompetent cells continue to be produced from proliferating progenitor cells in the bone marrow and thymus. Mature immunocompetent cells leave these primary immune organs and migrate via the blood to the secondary immune organs (e.g., spleen, lymph nodes, mucosal lymphoid tissues). The onset of functional competence depends on the specific parameter being measured and is also species-specific. Senescence of the immune responses

is not well understood, but it is clear that both innate and acquired immune responses to antigens are different in the last quartile of life. This failure of the immune response is due, in part, to a continual reduction in the production of newly formed cells, and to the decreased survival of long-lived cells in lymphoid tissues.

Humans: Immune system development in the human fetus generally begins with HSC formation in the yolk sac, which first appear to migrate at ~5 weeks, and is followed by seeding of lymphoid and myeloid lineage progenitor cells.^{122,123}

Mice: HSC first appear developmentally in intraembryonic splanchnopleuric mesenchyme surrounding the heart (or the aorto-gonadomesonephros) at ~8 days of gestation in mice.¹²⁴ These cells are found at essentially the same developmental stage in the extraembryonic blood islands of the yolk sac. Embryonic circulation is established by gestational day (GD) 8.5 in the mouse and it remains unclear to what extent there is exchange of cells from intraembryonic hematopoietic tissues to extraembryonic sites in any rodent species. Recent evidence, however, clearly demonstrates that the population of intraembryonic stem cells, but not those that appear in the yolk sac, contribute to sustained intraembryonic blood cell development and the emergence of the immune system in post-natal rodents.¹²⁴ For rats, dogs, and primates, there is no information available.

The concept that any of several dynamic changes associated with the developing immune system may provide periods of unique susceptibility to chemical perturbation has been previously reviewed.^{1,125,126} In particular, an understanding of these developmental landmarks has prompted some

to speculate about the existence of five critical windows of vulnerability in the development of the immune system:¹²⁶

1. The first “window”—a period of HSC formation from undifferentiated mesenchymal cells
 - a. Exposure of the embryo to toxic chemicals during this period could result in failures of stem cell formation, abnormalities in production of all hematopoietic lineages, and immune failure
2. The second “window”—characterized by migration of hematopoietic cells to the fetal liver and thymus, differentiation of lineage-restricted stem cells, and expansion of progenitor cells for each leukocyte lineage
 - b. This developmental window is likely to be particularly sensitive to agents that interrupt cell migration, adhesion, and proliferation
3. The third “window”—the establishment of bone marrow as the primary hematopoietic site and the establishment of the bone marrow and the thymus as the primary lymphopoietic sites for B-cells and T-cells, respectively
4. The fourth “window”—the initial period of perinatal immunodeficiency, and the maturation of the immune system to adult levels of competence
5. The fifth “window”—the subsequent period during which mature immune responses are manifest, and functional pools of protective memory cells are established

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,” data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Table 11.31).

TABLE 11.30
Summary of Immune System Development across Multiple Species

Parameter	Mouse (GD, %)	Dog (GD, %)	Primate (GD, %)	Human (GW, %)
Gestation	20 days	60–63 days ^a	155–165 days ^b	40 weeks
Stem cells appear	8 (40)	—	—	5 (12.5)
Fetal liver hematopoiesis	10 (50)	—	—	7 (17.5)
Splenic primordia	13 (65)	28 (45)	—	10 (25)
Spleen demarcation ^c	—	45 (72)	80 (50)	26 (65)
Thymic primordia	10 (50)	28 (45)	35 (22)	6 (15)
Bone marrow hematopoiesis	18 (90)	45 (72)	—	12 (30)
Mitogen proliferation ^d	17 (85)	50 (81)	—	12 (30)

Source: Adapted from Holsapple, M.P. et al., *Birth Defects Res. (Pt. B)*, 68, 321, 2003.

Note: Number in parentheses is the % of total length of gestation. GD, Gestational day; GW, gestational week.

^a Assume a gestational period of 62 days for later calculations.

^b Length of gestation affected by strain of primate; use 160 days for calculation.

^c Demarcation refers to the organization of spleen into distinct red and white pulp areas.

^d Proliferation in response to mitogens (PHA, Con A) by fetal thymocytes.

TABLE 11.31
Postnatal Development of the Immune System

Developmental Event	Postnatal Developmental Period (Days)	
	Human	Mice
B-Cell development ^a	Prenatal	Prenatal
T-Cell development ^a	Prenatal	Prenatal
NK-Cell development ^a	Prenatal	21
T-Dependent antibody response ^a	0	14 41–56 Adult level
T-Independent antibody response ^a	45–90	0 14–22 Adult level
Adult level IgG ^a	18–25	42–56

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

^a Holladay and Smialowicz.¹

RENAL

In a review article on comparative renal development, Zoetis and Hurtt¹²⁷ discuss the critical events in renal development involving both anatomical and functional aspects that occur within predictable time frames. Xenobiotics can affect either anatomical or functional development or both. Kidney maturation has been assessed by the size and distribution of the tubules and by the histological appearance of glomeruli.¹²⁸ Major renal anatomic developmental events occur in humans and other species prenatally (Table 11.32), with functional development of the major renal functions of glomerular filtration, urine concentration, acid base equilibrium, and urine volume control continuing into the postnatal periods. Completion of nephrogenesis marks the end of the anatomical development of the kidney. Nephrogenesis completes prenatally in humans, monkeys, mice, sheep, and guinea pigs, and postnatally in rats, dogs, and pigs (Table 11.33). Maturation of renal function occurs during different time frames for different species. Glomerular filtration can be detected as early as the first trimester of pregnancy and is important in tubular reabsorption of sodium and chloride ions that helps to maintain sodium balance in amniotic fluid. Concentrating ability develops prenatally in dogs and guinea pigs and postnatally in humans, rats, rabbits, and sheep. Acid–base equilibrium develops postnatally in all species reported including humans, rats, rabbits, and dogs. Control of urine volume also develops postnatally.

The design and interpretation of studies in prenatal and juvenile animals regarding renal development should include careful consideration of the variability in time points of maturation of both anatomical and functional developmental milestones between species.

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Tables 11.34 and 11.35).

TABLE 11.32
Onset of Kidney Development as Evidenced by Metanephros

Species	Metanephros (GD)	Total Gestation Period (Days)
Man	35–37	267
Macaque	38–39	167
Guinea pig	23	67
Rabbit	14	32
Rat	12.5	22
Mouse	11	19
Hamster	10	16
1. Chick	2. 6	3. 21

Source: Adapted from Zoetis, T. and Hurtt, M.E., *Birth Defects Res. (Pt. B)*, 68, 111, 2003 and references cited within.

TABLE 11.33
Timing of Completion of Nephrogenesis for Various Species

Species	Timing of Nephrogenesis Completion
Man	35 weeks gestation
Sheep	Before birth
Guinea pig	Before birth
Dog	Postnatal week 2
Pig	Postnatal week 3
Mouse	Before birth
Rat	Postnatal week 4–6

Source: Adapted from Zoetis, T. and Hurtt, M.E., *Birth Defects Res. (Pt. B)*, 68, 111, 2003 and references cited within.

TABLE 11.34
Renal—Functional

Developmental Event	Postnatal Developmental Period (Days)	
	Human	Rat
Glomerulo/nephrogenesis ^a	Prenatal	8–14
Adult GFR and tubular ^a	45–180	15–21

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

^a Snodgrass¹⁶⁶ and Travis.¹⁶⁷

TABLE 11.35
Renal—Anatomical

Developmental Event	Postnatal Developmental Period (Weeks)					
	Human	Dog	Rabbit	Rat	Mouse	Pig
Completion of nephrogenesis ^a	Prenatal week 35	2	2–3	4–6	Prenatal	3

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

^a Zoetis.¹⁶⁸

REPRODUCTIVE SYSTEM

Drugs and environmental chemicals have the potential to adversely affect the developing male and female reproductive system. The consequences of an exposure are influenced by the magnitude and duration of the exposure, the mechanism of action for the drug/chemical, the sensitivity of target tissue, and the critical windows in the development of the reproductive system. Table 11.36 is presented to define the postnatal age for phases of sexual development in the male organism and to indicate equivalent ages in selected common laboratory species, specifically the rat, Beagle dog, and nonhuman primates. The categories presented by the U.S. Department of Health and Human Services¹¹³ to aid in the design of clinical investigations of medicinal products are not included in the tables because they are not consistent with those used for the laboratory species. It is important to reiterate that the range of ages for a particular phase can vary somewhat depending on the organ systems under consideration and the species selected for the comparison. This follows from the observation that tissues and organs do not necessarily mature in the same sequence across all species or by the same overall stage of development.

Male

In a review article on comparative male reproductive system development, Marty et al.¹²⁹ discuss the critical events in human male reproductive system's development and compare it to other species. Topics discussed in the review include development of the testes, epididymides, the blood–testis barrier, anogenital distance, testicular descent, preputial separation, accessory sex glands (prostate and seminal vesicles), and the neuroendocrine control of the reproductive system.

Overall, there is evidence of similar patterns of male postnatal reproductive system development between humans and experimental animal models. However, a detailed examination of the developmental processes reveals pertinent cross-species differences. As research progresses, more thorough comparisons on the cellular and molecular level will become possible and the specific pathways targeted by chemicals can be identified. This knowledge will assist in the selection of sensitive and predictive animal models and further reduce uncertainty when extrapolating animal data to human risk.

Female

In a review article on comparative female reproductive system development, Beckman and Feuston¹³⁰ discuss the critical events in human female reproductive system's development and compare it to other species. Tables 11.37 and 11.38 present the timing of specific landmarks in the postnatal development of the female reproductive tract for the rat, dog, nonhuman primate, and human. Understanding the comparative development of the female reproductive system may aid in the selection of the most appropriate species for an investigation and it is important in the interpretation of results from investigations into the potential effects of chemical/compound exposures during the development of young animals. Maturation data for various species is presented in Table 11.39.

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Table 11.40).

TABLE 11.36
Species Comparison of the Male Reproductive System Development

	Human	Nonhuman Primate	Rat	Dog
Developmental stages	0–1 month neonatal 1 month–2 year infantile 2–12 year children 12–16 year adolescents		0–7 days neonatal 8–21 days infantile 21–35 days juvenile 35–(55–60) days peripubertal depending on sex	Phases of testis growth 1: 0–22 weeks 2: 22–36 weeks 3: 36–46 weeks
Puberty	12–14 years	2.5 years of age	Early puberty begins at PPS (~PND 43)	34–36 weeks, as defined by presence of ejaculated sperm

(continued)

TABLE 11.36 (continued)
Species Comparison of the Male Reproductive System Development

	Human	Nonhuman Primate	Rat	Dog
Spermatozoa	Spermatogonia increases sixfold between birth and 10 years of age, then increase at puberty. Mean age at spermatarche is 13.4 years; ejaculation possible during middle to late puberty	In the testis, spermatogonia becomes more numerous by the end of the first year Spermatozoa in the testis appears as early as 3 years with fertility starting at ~3.5 years	In seminiferous tubules—PND 45); in vas deferens—PND 58–59 Spermatogenesis is stimulated by increased testosterone and gonadotrophin production	In testis at 26–28 weeks of age First visible in the epididymis 26–28 weeks (beagles); first in ejaculate 32–34 weeks (fox terriers)
Leydig cells	Testosterone (T) producing cells, Leydig cells (LC), begin producing T ~7–8 weeks of gestation and ultimately come under control of placental gonadotrophin (human chorionic gonadotrophin [hCG]) Pituitary gonadotrophin synthesis begins at ~12 weeks of gestation after T production begins	LC are prominent in fetal life but decrease in number during the first year and dedifferentiate. By the end of 3 years, the LCs redifferentiate. T levels in immature males are ~30–250 ng/100 mL rising to 230–1211 ng/100 mL in mature males	Testosterone production begins during late gestation and decreases just prior to birth. During the infantile juvenile period (PND 8–35), the primary androgens produced include androstenedione, 5 α -androstanediol, and dihydrotestosterone, NOT testosterone but by ~PND 25 and onward testosterone becomes the primary androgen. Different androgen levels are primarily due to changes in steroidogenic enzyme levels or activity	First visible histologically GD 36–46. Testicular LH receptors begin to increase at 2 months, testicular maximum 12–24 months. Testicular T and DHT levels begin to increase at 6 months, plateau 12–24 months (beagles)
Sertoli cells	Sertoli cell (SC) differentiation and production of anti-Mullerian hormone (MIS) begin during the end of the first trimester and is triggered by an unknown mechanism mediated by the Y chromosome		Increase in number at ~GD 16 with peak division at GD 19 and cease division at BPND 14–16. Follicle-stimulating hormone (FSH) receptors on SCs increase markedly before birth and peaks ~PND 18 with a decline until adult levels met ~PND 40–50	First visible GD 36–46 (beagles). Divide up until 8 weeks postpartum, and stable thereafter

Source: Adapted from Marty, M.E. et al., *Birth Defects Res. (Pt. B)*, 68, 125, 2003 and references cited within.

TABLE 11.37
Phases of Sexual Development in the Female of Selected Common Laboratory Species

Phase	Rat	Dog (Beagle)	Nonhuman Primate
Neonatal	Birth to PND 7	Birth to 3 weeks	Birth to 3–4 months
Infantile	PND 8–21	3–5 weeks	Up to 29 months
Juvenile/prepubertal	PND 22–37	5 weeks to 6 months	Up to 43 months
Pubertal	PND 37–38	6–8 months	27–30 months

Source: Adapted from Beckman, D.A. and Feuston, M., *Birth Defects Res. (Pt. B)*, 68, 137, 2003 and references cited within.

TABLE 11.38
Landmarks in the Development of the Female Reproductive Tract for the Rat, Dog, Nonhuman Primate, and Human

Parameter	Species			
	Rat (Wistar or Sprague–Dawley, if Possible)	Dog (Beagle, if Possible)	Nonhuman Primate (<i>Macaca mulatta</i> , if Possible)	Human
Follicle maturation	Ovarian follicles become subjected to strong gonadotropin control during the second week of postnatal life	5–6 months: primary follicles show antrum formation	Not available	Follicles were seen in 86% of prepubertal girls and in 99% of pubertal girls
Ovulation	First ovulation on about PND 38 First ovulation on PND 29 First ovulation on PND 37	8–12 months 9–14 months	The postmenarcheal phase of development in the rhesus monkey is the result of a high incidence of an ovulatory and short luteal phase cycles	Ovulation—6 or more months after menarche. Regular ovulatory menstrual cycles—established several years later (12–24 month postmenarche) Ovarian function last ~30 years
Uterine maturation	Uterine glands first appear on PND 9 and increased until PND 15 Prepubertal: Small uterus, wet weight <100 mg with no intrauterine fluid Postpubertal: Larger uterus, wet weight greater than 200 mg Increase in uterine weight on PND 27	Puberty	Not available	Uterine growth begins before puberty
Puberty	Vaginal opening and first ovulation at ~5 weeks	8–12 months. 5–13 months (mean, 9 months) in 8–15 kg beagles	2.5–3 years, nipple growth, increase in perineal swelling and coloration	8–10 years: appearance of labial hair, initiation of breast enlargement Menarche at 8–14 years Menses at 12–13 years
Estrous cyclicity/ menarche (first estrus/menses)	~5 weeks	8–2 months	2–3 years	10–16.5 years; mean 13.4 years
Sexual maturity/ fertility	50 ± 10 days	6–12 months	2.6–3.5 years	11–16 years

Source: Adapted from Beckman, D.A. and Feuston, M., *Birth Defects Res. (Pt. B)*, 68, 137, 2003 and references cited within.

TABLE 11.39
Maturation Data for Various Species

	Human	Mouse	Rat	Rabbit	Dog	Monkey
Gestation (days)	267	20	22	32	63	167
Minimal breeding age (weeks)	728	7	10.5	28♀, 32♂	40♀, 32–40♂	218
Human to animal life span	1.0	44	33	12	7	4.4

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

TABLE 11.40
Species Comparison for Puberty

Development Event	Postnatal Development Period				
	Human (Years)	Rhesus Monkey (Years)	Dog (Days)	Mouse (Days)	Rats (Days)
Puberty ^a	11–12	2.5–3	180–240	35–45	40–60

Source: Adapted from FDA, Guidance for industry: Nonclinical safety evaluation of pediatric drug products, <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>, 2000.

^a DeSesso and Harris;¹⁶⁹ Marty et al.;¹²⁹ Beckman and Feuston;¹³⁰ and Lewis et al.¹⁴³

PULMONARY

In a review article on comparative lung development, Zoetis and Hurtt¹³¹ discuss the critical events in human lung growth and compare human lung development to that of other species. Developmental stages identified for the human lung are also found in other mammalian species reported in the literature. The timing of each developmental stage and the degree of lung development at birth vary widely between species.¹³² As an example, at birth the opossum lung is very primitive; rat and mouse lungs have no alveoli; kittens, calf, and humans have relatively few alveoli; and the lamb lungs are quite well developed. Postnatal development of the lung has also been studied in other species: guinea pig, hamster, dog, monkey, and humans. Table 11.41 presents the timing of each stage for several species.¹³³

The literature does not provide definitive evidence for the timing of the completion of alveolar development, and a weight-of-evidence approach is needed to address the safety of inhaled drugs intended for pediatric populations. Inhaled drugs intended to treat children over 2 years of age generally do not require extensive testing in juvenile animals. For children over the age of 2 years, the pulmonary safety of the drug is demonstrated in clinical trials by measuring lung function parameters during the course of the trial. The issue of local postnatal developmental toxicity for inhaled drugs is most important in children under 2 years of age. For the purpose of testing the safety of inhaled agents in pediatric populations <2 years of age, the rat and dog appear to be acceptable models.

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Table 11.42).

TABLE 11.41
Stages of Lung Development (in Gestation Days)

Species	Glandular	Canalicular	Saccular	Alveolar
Mouse	14–16	16.5–17.4	17.4 to —	PD 5 to —
Rat	13–18	19–20	21 to PD	PD 7–21
Rabbit	19–24	24–27	27 to —	—
Sheep	— to 95	95–120	120 to —	—
Human	42–112	112–196	196–252	252 to childhood

Source: Adapted from Zoetis, T. and Hurtt, M.E., *Birth Defects Res. (Pt. B)*, 68, 121, 2003.

TABLE 11.42
Lung Growth in Humans and Rats (Numbers Represent the Fold Change from Newborn to Adult)

Pulmonary Parameter	Human	Rat
Lung volume (mL)	23.4	23.5
Parenchyma airspace volume (mL)	30.2	26.9
Septal volume (mL)	13.5	13.6
Alveolar surface area (m ²)	21.4	20.5
Capillary surface area (m ²)	23.3	19.2

Source: Zeltner, T.B. et al., *Morphom. Respir. Physiol.*, 67, 247, 1987.

BONE

In a review article on comparative bone development, Zoetis et al.²⁵ discuss the critical events in human postnatal bone growth and compare human bone development to that of other species. Bone is a dynamic connective tissue whose postnatal development in all species reflects its multiple tasks within the body (e.g., mechanical support as part of the locomotor apparatus, protecting vital organs, reservoir for calcium and phosphorus for metabolic pathways associated with mineral homeostasis, production of blood elements).

Important characteristics of postnatal bone growth are expressed in different types of bone. Each bone has a unique shape that reflects a specific function and when this function started to develop. Growth patterns of specific bones exhibit important characteristics of postnatal bone development. The critical milestones in postnatal bone growth and development are the appearance of secondary ossification centers (Table 11.43), longitudinal bone growth and the epiphyseal growth plate, the fusion of secondary ossification centers, diametric bone growth, and bone vascularity.

In the FDA “Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products,”¹¹³ data are provided with the comparative developmental timing that was considered current at the time this guidance was developed (Table 11.44).

TABLE 11.43
Age of Appearance and Fusion of Secondary Ossification Centers in the Humerus and Femur

	Age			
	Appearance		Fusion	
	Proximal Epiphysis	Distal Epiphysis	Proximal Epiphysis	Distal Epiphysis
Humerus				
Human	Gestation week 36 to 4 years	6 months to 10 years	12–20 years	11–19 years
Monkey	Birth	Birth to 1 month	4–6 years	1.75–4.5 years
Dog	1–2 weeks	2–9 weeks	10–12 months	6–8 months
Rabbit	1 day	1 day	32 weeks	32 weeks
Rat	8 days	8–30 days	52–181 weeks	31–158 days
Mouse	5–10 days	5–19 days	6–7 weeks	3 weeks
Femur				
Human	1–12 years	Gestation week 36–40	11–19 years	14–19 years
Monkey	Birth to 6 months	Birth	2.25–6 years	3.25–5.75 years
Dog	1 week to 4 months	2–4 weeks	6–13 months	8–11 months
Rabbit	1–5 days	1 day	16 weeks	32 weeks
Rat	21–30 days	8–14 days	78–156 weeks	15–162 weeks
Mouse	14–15 days	7–9 days	13–15 weeks	12–13 weeks

Source: Adapted from Zoetis, T. et al., *Birth Defects Res. (Pt. B)*, 68, 86, 2003.

TABLE 11.44
Postnatal Fusion of Secondary Ossification Center

Developmental Event	Postnatal Developmental Period					
	Human (Years)	Rhesus Monkey (Years)	Dog (Days)	Rabbit (Days)	Rat (Days)	Mouse (Days)
Fusion of Secondary Ossification Centers ^a						
Femur distal epiphysis	14–19	3–6	0.7–0.9	32	15–162	12–13

^a Zoetis.¹⁶⁸

DESIGNING A JUVENILE TOXICITY STUDY PROTOCOL

Juvenile toxicity studies are used to bridge the “gap” between the *in utero* exposure (GD 6–20) and lactational exposure (PND 0–20) of the offspring in the pre- and postnatal toxicity study and the ~7–8 weeks of age where General Toxicity Studies routinely begin exposures and assessments.

Juvenile toxicity studies combine the usual adult toxicity end points and internal exposure determinations with the appropriate developmental assessments. Juvenile toxicity studies are designed on a case-by-case basis.

The study design determines the conclusions that may be drawn from collected data. Species selection, age at initiation of treatment, litter composition, dosage levels, duration of treatment, ages at various assessments, and end points measured during and after treatment are all major considerations in designing these studies (Table 11.45 and Figure 11.9). The most frequent functional end points in juvenile toxicity studies are behavioral and reproductive performance while other organ system functional assessments may be more relevant for some agents (e.g., kidney function for ACE inhibitors, immune function for biologics, etc.).

TABLE 11.45
Species Selection

Species/Parameter	Rat	Dog	Monkey
Target organ toxicity	Good	Good	Good
Exposure/toxicokinetics	Fair	Good	Good
Development staging	Good	Good	Good
Human matching	Poor	Poor	Good
Background data	Good	Fair	Poor
CNS functional data	Good	Poor	Poor
Reproductive development	Good	Impractical	Impractical
Statistical analysis	Good	Poor	Poor

Source: Adapted from Bailey, G. and Schaepdrijver, L.D., *ILSI/HESI Workshop: The Value of Juvenile Toxicity Studies: Introduction and 'Warm up,'* Washington, DC, April 5–6, 2010 (unpublished workshop).

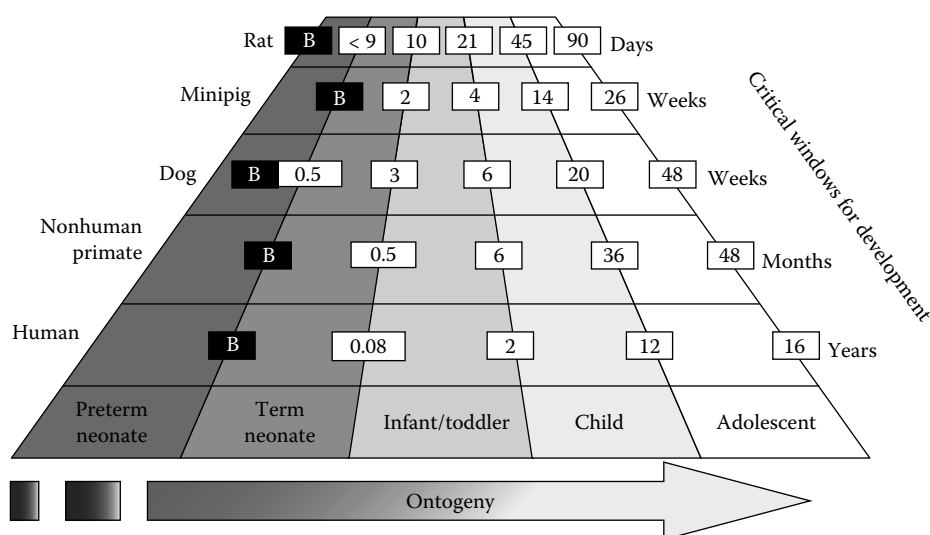


FIGURE 11.9 Comparative age categories based on overall CNS and reproductive development. (From Buelke-Sam, J., Comparative age categories in juvenile toxicity studies, Presentation at the FDA/CDER Office of New Drugs “Juvenile Animal Study Workshop,” Silver Spring, MD, May 2011.)

LITTER COMPOSITION (RATS)

Depending upon the pediatric age classification, juvenile toxicity studies often initiate dosing prior to weaning. Therefore, dosing must be performed in the litter setting. The litter is the largest contributor to physical and behavioral variability in rodents and actually becomes more so as animals age.¹³⁴ Litter is the sampling unit for testing/statistics in DART studies and must be accounted for in the design of juvenile toxicity studies as well.

There are several methods of litter composition: (1) within-litter; (2) between litter; (3) one pup per sex per litter; (4) fostering; and (5) split litter. For simplicity, litter size and sex ratio for this discussion will be set at eight pups (four males and four females) and the study design requires four groups (one control and three treated groups [low, mid, and high]) of 12 per sex per group. Each method of litter composition has its advantages and disadvantages (logistically, cross-contamination possibility,

and statistically). The within-litter design has the litter's animals assigned to each treatment group (control group—male 1 and female 1; low group—male 2 and female 2; mid group—male 3 and female 3; and high group—male 4 and female 4). While statistically sound, it is logistically difficult; each pup requires dosing from a different dose level causing difficulties for the technicians in identifying the pups and administering the dosage correctly. This method is also fraught with cross-contamination issues. The between-litter design has all pups in the litter treated with the same dosage, while minimizing technician effort because dosing is less complicated and there would be no contamination issues, it is not statistically optimal due to the well-known litter effect (siblings tend to respond more similarly than nonsiblings). The one pup per sex per litter design eliminates the litter effect and therefore is statistically acceptable. There would be no contamination issues and the technical effort would be simplified; however, the drawback is the number of litters required (48 litters) and number of pups

not selected and therefore discarded (288 pups). The one pup per sex per litter design would be very difficult to justify to the Institutional Animal Care and Use Committee. The fostering design has each litter composed of pups from other litters without using any same sex siblings and all pups within the new fostered litter receive the same treatment. Cross fostering eliminates the litter effect and therefore is statistically acceptable. There would be no contamination issues and the daily technical effort would be simplified. However, the cross-fostering procedure (discussed later) is a time-consuming and labor-intensive process. Split litter design is a variation of the within-litter design and has been used when the number of treatment groups exceeds the number of pups per litter, if for example the study design requires seven groups then the litter size would be too great large to have each litter have all of the treatment groups.

CROSS FOSTERING

The following discussion details a procedure followed for cross-fostering litters of rat litters on PND 1. On Day 20 of presumed gestation, parturition observations (three times daily) are begun. When a dam has completed delivering its litter, the time is recorded on the parturition observations sheet. If the beginning of parturition is observed, that is, the birth of the first pup, this is deemed PND/LD 0. If a dam delivers overnight and delivery is first observed as complete at the morning parturition observation, then it is taken that parturition began prior to midnight and the day of observed completion is deemed as PND/LD 1. If a dam begins delivery in the morning after the first parturition observation and delivery is completed that day, then day of observed completion is deemed to be PND/LD 0.

After the morning parturition observations are completed and a list of litters to be cross-fostered on that day (PND 1) is

determined, the pups in each original birth litter are separated by sex and counted. This information is entered on the appropriate form including details of any pups that the technicians feel should be excluded from the cross fostering (e.g., runts, deformed pups, etc.) and the pups are returned to their mothers.

The cross-fostering plan documentation is made. The details of the birth litters and the pups available for cross fostering are recorded and this information is verified by a second person. The pups are selected and arranged into *virtual* foster litters with no pup remaining with its birth mother and no same sex siblings in each litter. While it is acceptable to have one male and one female sibling in the fostered litter, ideally there will be no siblings in the same litter. This cross-fostering plan documentation is then verified by a second person and approved by the study director or designee prior to use (Table 11.46).

Each litter is removed from its home cage and placed in containers by sex labeled with the birth dam's number and the sex of the pups. When all litters have been sorted, then the *physical* cross fostering is performed. Using a partitioned box labeled with the foster dam's number and with each partition labeled with the pup number within the foster litter and using the cross-fostered litters sheet, the selected pups are removed from the birth litter containers and placed in the appropriate space in the foster litter box.

Once the entire foster litter is composed, the pups are tattooed, weighed, sexed, and placed in their foster mother's cage. The placing of the foster litter in the foster dam's cage is achieved by removing the foster dam, placing the foster pups in the cage, rolling the pups in the bedding to acquire some of the scent of the foster dam and the home cage, and then returning the foster dam to the cage. Utilization of this methodology for cross-fostering pups on PND 1 has resulted in no pup rejections by the dams in over 450 cross-fostered litters during the past year.

TABLE 11.46
Natural Litters and Cross-Fostering Plan

Natural Litters

Dam	Male Pups							Female Pups						
100	101	102	103	104	105	106	107	151	152	153	154	155	156	
200	201	202	203	204	205			251	252	253	254	255	256	257
300	301	302	303	304	305	306		351	352	353	354	355		
400	401	401	403	404	405			451	452	453	454	455	456	457
500	501	502	503	504	505	506	507	551	552	553	554	554		
600	601	602	603	604	605	606		651	652	653	654	655	656	657

Cross-Fostered Litters (No Same Sex Siblings Allowed)

Foster Dam Number	Foster Pup Number									
	Male Pups					Female Pups				
100	201	301	401	501	601	251	351	451	551	651
200	102	302	402	502	602	152	352	452	552	652
300	103	202	403	503	603	153	252	453	553	653
400	104	304	203	504	604	154	354	253	554	654
500	105	305	405	205	605	155	355	455	255	655
600	106	306	406	506	206	156	356	456	556	256

PHARMACOKINETICS AND TOXICOKINETICS

It is recognized that the collection of blood samples to obtain a full kinetic profile of a test compound under study in juvenile animals might sometimes be impractical. However, sampling at a few time points, using pooled terminal blood samples from pups aged PND 1–6 or a single terminal blood collection from pups aged PND 7–21 should be performed to obtain an estimate of basic kinetic characteristics (e.g., C_{max} and AUC).

Toxicokinetics should also be considered to confirm appropriate exposure levels in different treatment groups. Under special circumstances, data on absorption, distribution, metabolism, and/or excretion in juvenile animals may be valuable for studying a specific safety concern. Such detailed data will only be necessary if it is anticipated (based on *in vitro* data, scientific rationale, etc.) that the pharmacokinetic and toxicokinetic characteristics of the juvenile animal model(s) are comparable to the human situation at the stage of development so that the data can be used in efficacy and/or safety evaluation.

ANIMAL NUMBER REQUIREMENTS

BASED ON AGE AT SAMPLING

The number of F_0 animals required for a juvenile toxicity study depends on its design. The following narrative will give the reader a method of estimating the minimum number necessary to allow for the delivery of a sufficient number of litters for cross fostering that meet the guideline limitations, taking into account Historical Control Data. Enough litters should be available to assure that a sufficient number of juvenile male and female pups are available for allocation to the treatment groups.

The normally expected pregnancy rate for Sprague–Dawley rats from a vendor (Charles River Laboratories) is ~90% (with a range of 59%–100%, as per Historical Control Data¹³⁵). Litters with fewer than 10 total pups (i.e., including both males and females) are excluded from the study so that all dams are capable of supporting cross-fostered litters consisting of 4 or 5 male and 4 or 5 female pups and so the pup body weights are consistent (pups from smaller litter sizes generally are heavier than pups from larger litters). Based on the recent pre- and postnatal development studies and pubertal development studies at the author's testing facility, ~92% of the pregnancies resulted in litters with greater than 10 total pups. Litters with 10 pups (but with less than 3 male or 3 female pups) are also excluded from the study. At the testing facility, ~85% of the litters had sufficient number of males and/or females.

Cross fostering and standardizing the litter size to 10 (5 male:5 female) pups on PND 1 is considered to be optimal to maintain consistent litters and to avoid known litter effects.

For example, if the study design requires 20 F_1 pups/sex/group with four groups to be treated preweaning 80 F_1 pups/sex will be required. By using cross-fostered litters where there is no more than one male and one female from any original dam, the

entire litter can be dosed at one dose level. Four cross-fostered litters consisting of 5 males and 5 females each will supply the 20 F_1 pups/sex/group. Therefore, 16 litters will be required. To get the correct number of pregnant females with the appropriate litter size and sex ratio, the following formula is used: multiply the number of cross-fostered litters required (16) by the number of litters that have the proper sex ratio (1.15; 100% plus the 15%), number of litters with the appropriate size (1.08), and the number of dams ordered that will deliver (1.1). At each step, the number must be rounded up to the next whole animal.

$$\text{Number of animals to order} = 16 \times 1.15 = 18.4$$

$$(\text{round up to } 19); 19 \times 1.08 = 19.2 (\text{round up to } 20)$$

$$20 \times 1.1 = 22.0$$

Therefore, the number of presumed pregnant dams required to generate 16 cross-fostered litters is 22. If there is a staggered start, these factors need to be used to generate the numbers for each replicate. For example, two replicates of eight cross-fostered dams will require

$$\text{Number of animals to order} = 8 \times 1.15 = 9.2$$

$$(\text{round up to } 10); 10 \times 1.08 = 10.8 (\text{round up to } 11);$$

$$11 \times 1.1 = 12.2 (\text{round up to } 13)$$

Therefore, each replicate will require 13 for a total of 26 or 4 more than if done as a single set.

Another issue that must be accounted for in pups that are <8 weeks of age is blood volume available for toxicokinetics, clinical chemistry, hematology, and coagulation evaluations (see the sections on blood volumes based on age and weight and number of samples later). For very young animals, pooling of blood samples may be required to get sufficient sample size. For most studies, separate toxicokinetic and clinical pathology animals will be required.

BLOOD VOLUMES BASED ON AGE AT SAMPLING

Blood volumes differ based on age, body weight, and sex. The volume of blood that can be obtained from small rodent pups is usually less than that would be predicted by the standard total blood volume calculation based on weight. Blood collection from preweaning pups is usually done as a terminal collection often by decapitation (see later). Blood can be collected from the orbital fossa or from the vena cava in older postweaning pups. Other sites are more difficult due to vessel diameter and friability. Multiple blood sampling within a single day or a few days may not be possible with early postweaning pups (PND 21–28). This topic is discussed in detail in Diehl et al.¹³⁶ and based on the blood volume calculation method in this article, the total blood volumes that can be expected are shown in Tables 11.47 and 11.48.

TABLE 11.47
Blood Volume in Male and Female Rats based on Age

PND	7	14	21	28	35	42	49	56	63	70	77	84
Mean body weight males (g)	16.8	34	54.9	92.6	150.8	212.9	273.5	329.4	379.5	424.6	458.7	489
Mean body weight females (g)	15.8	32.5	52.6	86.2	131	169.1	196.5	222	242.3	260.9	274.1	287.3
Blood Volume (mL)^a												
Total males	1.08	2.18	3.51	5.93	9.65	13.63	17.50	21.08	24.29	27.17	29.36	31.30
Total females	1.01	2.08	3.37	5.52	8.38	10.82	12.58	14.21	15.51	16.70	17.54	18.39
7.5% Males	0.08	0.16	0.26	0.44	0.72	1.02	1.31	1.58	1.82	2.04	2.20	2.35
7.5% Females	0.08	0.16	0.25	0.41	0.63	0.81	0.94	1.07	1.16	1.25	1.32	1.38
10.0% Males	0.11	0.22	0.35	0.59	0.97	1.36	1.75	2.11	2.43	2.72	2.94	3.13
10.0% Females	0.10	0.21	0.34	0.55	0.84	1.08	1.26	1.42	1.55	1.67	1.75	1.84
15.0% Males	0.16	0.33	0.53	0.89	1.45	2.04	2.63	3.16	3.64	4.08	4.40	4.69
15.0% Females	0.15	0.31	0.50	0.83	1.26	1.62	1.89	2.13	2.33	2.50	2.63	2.76
20.0% Males	0.22	0.44	0.70	1.19	1.93	2.73	3.50	4.22	4.86	5.43	5.87	6.26
20.0% Females	0.20	0.42	0.67	1.10	1.68	2.16	2.52	2.84	3.10	3.34	3.51	3.68

^a Formula (64 mL/kg × body weight in kilograms) = total blood volume (mL).

TABLE 11.48
Blood Volume in Male and Female Rats based on Body Weight

Rat Body Weight (g)	15	50	75	100	125	150	200	250	300	350	400	450	500	550	600
Blood Volume (mL)															
Total	0.96	3.2	4.8	6.4	8	9.6	12.8	16	19.2	22.4	25.6	28.8	32	35.2	38.4
7.5%	0.072	0.24	0.36	0.48	0.6	0.72	0.96	1.2	1.44	1.68	1.92	2.16	2.4	2.64	2.88
10%	0.096	0.32	0.48	0.64	0.8	0.96	1.28	1.6	1.92	2.24	2.56	2.88	3.2	3.52	3.84
15%	0.144	0.48	0.72	0.96	1.2	1.44	1.92	2.4	2.88	3.36	3.84	4.32	4.8	5.28	5.76
1. 20%	0.192	0.64	0.96	1.28	1.6	1.92	2.56	3.2	3.84	4.48	5.12	5.76	6.4	7.04	7.68

BLOOD COLLECTION FROM YOUNG RATS

Procedure for terminal blood collection from young rats (ca. PND 7): The animal is placed in a plastic restraint cone (e.g., Decap Cone) so that the forelimbs are depressed against the body toward the tail. The plastic cones are used for restraint purposes to minimize stress on the animal from handling and to ensure correct placement of the animal in the scissors. The animal is anesthetized by administration of ~0.2 mL of 39% sodium pentobarbital via intraperitoneal injection (an additional dose may be given as needed to ensure sufficient anesthesia is achieved). The level of anesthesia is checked by testing for absence of tail and toe pinch reflex. Bring the animal to the blood collection area and decapitate with a single, strong, cut of the scissors. Hold the animal's torso over the collection vial to drain the blood (a funnel may be used to assist with collection into the vial). Thoroughly clean the scissors, funnel, and surrounding area to avoid cross-contamination prior to proceeding to the next animal.

DOSING VOLUMES BASED ON BODY WEIGHT

Administration of substances to laboratory animals requires careful consideration and planning to optimize delivery of the agent to the animal while minimizing potential adverse experiences from the procedure. This is especially true for juvenile animals. For all species, many different routes are available for administration of substances but the age that they can initiate dosing varies based on animal size and age (Table 11.49). The research team and IACUC members should be aware of reasons for selecting specific routes and of training and competency necessary for personnel to use these routes effectively. Once a route is selected, issues such as volume of administration, site of delivery, pH of the substance, and other factors must be considered to refine the technique. Inadequate training or inattention to detail during this aspect of a study may result in unintentional adverse effects on experimental animals and confounded results. Guidance on the routes of administration and appropriate volumes for administration plus other factors to consider can be found in the article by Turner et al.¹³⁷

TABLE 11.49
Earliest Starting Administration based on Route
of Administration and Species

Route of Administration	Rat (PND)	Mice (PND)	Rabbit (PND)	Dog (PND)	Minipig (PND)
Oral gavage	1	4	14	1	1
Subcutaneous	1	1	6	1	1
Intramuscular	1	1	6	1	1
IV bolus (repeated)	4	7	6	1	7 ^a
IV infusion	21	21	28	56	7 ^a
Inhalation (whole body)	4	4	6	10	2–7
Inhalation (nose only)	21	21	28	4 ^b	4 ^b
Dermal ^c	21	21	28	42	28

Source: Personal experience and adapted from Hoberman, A.M. and Lewis, E.M., eds., *Pediatric Non-Clinical Drug Testing: Principles, Requirements and Practices*, John Wiley & Sons, Hoboken, NJ, 2010.

^a Difficult because there are no easily accessible veins (vascular implant port can be surgically installed after PND 7).

^b Not recommended in preweaning animals.

^c Masks can be fit as young as PND 4 but restraint issues arise.

DEVELOPMENTAL LANDMARKS AND REFLEX ONTOGENY

The onset of various developmental landmarks can be used to assess postnatal development. Sexual maturation landmarks (preputial separation and vaginal patency) are required or recommended in juvenile toxicity studies, where the offspring are raised to adulthood. Additional developmental landmarks can be assessed, including pinna detachment, hair growth (pilation), incisor eruption, eye opening (pups are born blind with eyelids closed), nipple development, and testis descent. The examinations must begin prior to the landmark's historical day of onset and continue daily until each animal in the litter meets the criterion.¹³⁸ Data for each day's testing should be expressed as the number of pups that have achieved the criterion for each developmental landmark, divided by the total number of pups tested in the litter. Auditory startle and pupillary constriction tests are conducted on PND 21 only. Data for the auditory startle test and pupillary constriction tests should be expressed as the number of pups that have achieved the criterion, divided by the total number of pups tested in the litter. The PND listed in Table 11.50 for each developmental landmark were compiled from several

sources and are subject to variability between laboratories and subtle differences in assessment of the criteria.^{138–140}

Balanopreputial Separation

In rats, balanopreputial separation is considered to result from balanopreputial membrane cornification, which leads to the detachment of the prepuce from the glans penis in the rat. Preputial separation occurs dorsolaterally and then ventrally on the penis and down the shaft of the penis.¹⁴¹ The prepuce remains attached to the glans penis on its ventral surface by the frenulum. (Note: The process of development of the prepuce in humans is different from that of the rat.) Male rodents are examined for balanopreputial separation beginning on PND 22 (mouse), PND 27 (hamster), or PND 35–40 (rat). Published preputial separation age ranges PND 41–46,^{141–143} depending on the observation criterion with the typical mean around PND 43.5 ± 2.5.

Each male rodent is removed from its cage and held in a supine position. Because manipulation of the prepuce can accelerate the process of preputial separation, the males must be examined gently. Gentle digital pressure is applied to the sides of the prepuce, and the criterion is met when the prepuce completely retracts from the head of the penis. The foreskin can be attached along the shaft of the penis, but it cannot be attached to the opening of the urethra. Each male rodent is examined daily until acquisition or until PND 55, whichever is earlier. Body weight should be recorded on the day the criterion is met.

Vaginal Patency

After canalization of the vagina occurs in rats, the vaginal opening remains covered by a septum or membrane. The age when the septum is broken or no longer evident is described as the age of vaginal patency, and vaginal patency is the most readily determined marker for puberty in rats.¹⁴¹ Published Sprague–Dawley rat vaginal patency values range from PND 30.8 to 38.4^{144,145} with typical mean being PND 32.3 ± 0.5. Female rats are examined beginning a few days prior to the expected age of maturation (e.g., PND 25–28), continuing until the criterion for patency has been achieved or until PND 43, whichever comes first. The female is removed from the cage and held in a supine position, exposing the genital area. Pressure is gently applied to the side of the vaginal opening to see if the septum or membrane remains. When the membrane is present, the area has a slight “puckered” appearance; however, when the membrane has broken, the vaginal opening is about the size of a pinhead. The criterion has been met when the vagina is completely open. Body weight should be recorded on the day the criterion is met.

TABLE 11.50
Prewaning Developmental Landmarks in Rats

Developmental Landmark	Methodology	Evaluation Initiation	Acquisition Range	References
Pinna unfolding	Point of a pinna (ear flap) examined to determine detachment from a head	PND 1	PND 2–3	142,143
Hair growth	Pups examined until bristles appear on the dorsal surface of all pups in the litter	PND 1	PND 1–5	143
Incisor eruption	Eruption through the gum of either an upper or lower incisor	PND 7	PND 8–16, mean PND 11	142,143,173
Eye opening	Pups examined break in the membrane connecting the upper and lower eyelids	PND 10	PND 11–18, Mean PND 13 or 14	142,143
Nipple retention	Males in each litter examined for the presence of areolae and/or nipples by brushing the hair coat against the nap	PND 11	PND 12–13	

EUTHANASIA

The AVMA Guidelines on Euthanasia¹⁴⁶ are recognized by government regulators and the animal care and use community as the gold standard for acceptable procedures and agents for euthanizing a broad spectrum of animal species. The AMVA panel used the following criteria in evaluating methods of euthanasia: “(1) ability to induce loss of consciousness and death without causing pain, distress, anxiety, or apprehension; (2) time required to induce loss of consciousness; (3) reliability; (4) safety of personnel; (5) irreversibility; (6) compatibility with requirement and purpose; (7) emotional effect on observers or operators; (8) compatibility with subsequent evaluation, examination, or use of tissue; (9) drug availability and human abuse potential; (10) compatibility with species, age, and health status; (11) ability to maintain equipment in proper working order; and (12) safety for predators/scavengers should the carcass be consumed.”

The Report of the AVMA Panel on Euthanasia provides limited recommendations for the euthanasia of prenatal or neonatal animals in the section entitled “Guidelines for the Euthanasia of Mouse and Rat Fetuses and Neonates.” The following guidelines are suggested to assist individual Animal Care and Use Committees at the NIH in reviewing proposals which involve the use of rodent fetuses or neonates.

Fetuses

1. *Fetuses up to 14 days in gestation:* Neural development at this stage is minimal and pain perception is considered unlikely. Euthanasia of the mother or removal of the fetus should ensure rapid death of the fetus due to loss of blood supply and nonviability of fetuses at this stage of development.¹⁴⁷
2. *Fetuses 15 days in gestation to birth:* The literature on the development of pain pathways suggests the possibility of pain perception at this time.

Whereas fetuses at this age are resistant to inhalant anesthetics including CO₂, euthanasia may be induced by the skilful injection of chemical anesthetics. Decapitation with surgical scissors, or cervical dislocation is acceptable physical methods of euthanasia. Rapid freezing, without prior anesthesia, as a sole means of euthanasia is not considered to be humane. Animals should be anesthetized prior to freezing. When chemical fixation of the whole fetus is required, fetuses should be anesthetized prior to immersion in or perfusion with fixative solutions. Anesthesia may be induced by hypothermia¹⁴⁸ of the fetus, injection of the fetus with a chemical anesthetic, or deep anesthesia of the mother with a chemical agent that crosses the placenta, example, pentobarbital. The institute veterinarian should be consulted for considerations of fetal sensitivity to specific anesthetic agents. When fetuses are not required for study, the method chosen for euthanasia of a pregnant mother must ensure rapid death of the fetus.

Neonates

1. *Up to PND 14:* Acceptable methods for the euthanasia of neonatal mice and rats include injection of chemical anesthetics (e.g., pentobarbital), decapitation, or cervical dislocation. Additionally, these animals are sensitive to inhalant anesthetics, example, halothane or isoflurane (used with appropriate safety considerations). Immersion in liquid nitrogen may be used only if preceded by anesthesia. Similarly, anesthesia should precede immersion or perfusion with chemical fixatives. Anesthesia may be induced by inhalant or

injectable anesthetics; the institute veterinarian should be consulted for appropriate agents and dosages. Alternatively, when adequately justified, hypothermia¹⁴⁸ may be used to induce anesthesia in PND 6 pups or less.

2. *Older than PND 14*: Follow guidelines for adults.

In all cases, the person performing the euthanasia must be fully trained in the appropriate procedures.

CONCLUSION

Juvenile toxicity studies aim at bridging potential data gaps between the pre- and postnatal toxicity studies (Segment III) where the offspring are potentially exposed *in utero* and through maternal milk until weaning and the repeat-dose toxicity studies (in young adult animals). There is no STANDARD juvenile toxicity study design; however, the study designs are becoming more standardized. The study design needs to consider different stages and pace of development of the organ systems at risk in human and the animal species equivalent. Species, age at initiation and duration of treatment, “within” versus “between” litter designs, ages at various assessments, and end points measured during and after treatment are all major considerations in designing studies on a case-by-case basis. The rat is the species of choice and juvenile toxicity studies will involve direct dosing of pups.

Juvenile toxicity studies combine standard adult toxicity end points, TK exposure determinations, and appropriate developmental assessments. The most frequent functional end points are behavioral assessments and reproductive performance. Other organ system functional assessment may be required by the Regulatory Agency because they are considered more relevant for some agents (e.g., kidney function for ACE inhibitors). Biologics will require immunological assessments.

And always remember that the conduct of a preliminary juvenile study is highly recommended and NEVER run the Pivotal study until there is regulatory consultation and agreement on the study design and species.

REFERENCES

- Holladay, S.D. and Smialowicz, R.J. (2000) Development of the murine and human immune system: Differential effects of immunotoxicants depend on time of exposure. *Environ Health Perspect*, 108(Suppl 3):463–473.
- Burns-Naas, L.A., Hastings, K.L., Ladics, G.S., Makris, S.L., Parker, G.A., and Hopsapple, M.P. (2008) What's so special about the developing immune system? *Int J Toxicol*, 27:223–254.
- Adams, J., Barone, S., Jr., LaMantia, A., Philen, R., Rice, D.C., Spear, L., and Susser, E. (2000) Workshop to identify critical windows of exposure for children's health: Neurobehavioral work group summary. *Environ Health Perspect*, 108(Suppl 3):535–544.
- Farwell, J.R., Lee, Y.J., Hirtz, D.G., Sulzbacher, S.I., Ellenberg, J.H., and Nelson, K.B. (1990) Phenobarbital for febrile seizures—Effects on intelligence and on seizure recurrence. *N Engl J Med*, 322:364–369.
- Fonseca, N.M., Sell, A.B., and Carlini, E.A. (1976) Differential behavioral responses of male and female adult rats treated with five psychotropic drugs in the neonatal stage. *Psychopharmacologia*, 46:253–268.
- Diaz, J., Schain, R.J., and Bailey, B.G. (1977) Phenobarbital-induced brain growth retardation in artificially reared rat pups. *Biol Neonate*, 32:77–82.
- Towfighi (1980) In: Hexachlorophene Spencer and Scaumburg, eds., *Experimental and Clinical Neurotoxicology*. Baltimore, MD: Williams & Williams, pp. 440–455.
- Skovranek, J., Ostadal, B., Pelouch, V., and Prochazka, J. (1986) Ontogenetic differences in cardiac sensitivity to verapamil in rats. *Pediatr Cardiol*, 7:25–29.
- Boucek, R.J., Jr., Shelton, M., Artman, M., Mushlin, P.S., Starnes, V.A. et al. (1984) Comparative effects of verapamil, nifedipine, and diltiazem on contractile function in the isolated immature and adult rabbit heart. *Pediatr Res*, 18: 948–952.
- Mares, P., Kubova, H., and Czuczwar, S.J. (1994) Aminophylline exhibits convulsant action in rats during ontogenesis. *Brain Dev*, 16(4): 296–300.
- Yokoyama, H., Onodera, K., Yagi, T., and Iinuma, K. (1997) Therapeutic doses of theophylline exert proconvulsant effects in developing mice. *Brain Dev*, 19:403–407.
- Vorhees, C.V., Ahrens, K.G., Acuff-Smith, K.D., Schilling, M.A., and Fisher, J.E. (1994) Methamphetamine exposure during early postnatal development in rats: I. Acoustic startle augmentation and spatial learning deficits. *Psychopharmacology*, 114:392–401.
- Greely, G.H. and Kizer, J.S. (1980) The effects of chronic methylphenidate treatment on growth and endocrine function in the developing rat. *J Pharmacol Exp Ther*, 215:545–551.
- Pizzi, W.J., Rode, E.C., and Barnhart, J.E. (1987) Differential effects of methylphenidate on the growth of neonatal and adolescent rats. *Neurotoxicol Teratol*, 9:107–111.
- Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J. et al. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science*, 283:70–74.
- Sidhu, R.S., Del Bigio, M.R., Tuor, U.I., and Seshia, S.S. (1997) Low-dose vigabatrin (gamma-vinyl GABA)-induced damage in the immature rat brain. *Exp Neurol*, 144:400–405.
- Wegerer, V., Moll, G.H., Bagli, M., Rothenberger, A., Ruther, E., and Huether, G. (1999) Persistently increased density of serotonin transporters in the frontal cortex of rats treated with fluoxetine during early juvenile life. *J Child Adolesc Psychopharmacol*, 9:13–24.
- Stahlmann, R., Chahoud, I., Thiel, R., Klug, S., and Forster, C. (1997) The developmental toxicity of three antimicrobial agents observed only in nonroutine animal studies. *Reprod Toxicol*, 11:1–7.
- Brenner, D.J., Elliston, C.D., Hall, E.J., and Berdon, W.E. (2001) Estimated risks of radiation-induced fatal cancer from pediatric CT. *Am J Roentgenol*, 176(2):289–296.
- Hall, E.J. (2002) Lessons we have learned from our children: Cancer risks from diagnostic radiology. *Pediatr Radiol*, 32(10):700–706.
- Rice, D. and Barone, S., Jr. (2000) Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environ Health Perspect*, 108(Suppl 3):511–533.

22. Radde, I.C. (1985) Mechanism of drug absorption and their development. In: Macleod, S.M. and Radde, I.C., eds., *Textbook of Pediatric Clinical Pharmacology*. Littleton, MA: PSG Publishing Co., pp. 17–43.
23. Miyawaki, T., Moriya, N., Nagaoki, T., and Taniguchi, N. (1981) Maturation of B-cell differentiation ability and T-cell regulatory function in infancy and childhood. *Immunol Rev*, 57:61–87.
24. Zoetis, T. and Walls, I. (2003) *Principles and Practices for Direct Dosing of Pre-Weaning Mammals in Toxicity Testing and Research*. Washington, DC: ILSI Press, p. 11 and 13.
25. Zoetis, T., Tassinari, M.S., Bagi, C., Walthall, K., Mark, E., and Hurtt, M.E. (2003) Species comparison of postnatal bone growth and development. *Birth Defects Res (Pt B)*, 68:86–110.
26. Walthall, K., Cappon, G.D., Hurtt, M.E., and Zoetis, T. (2005) Postnatal development of the gastrointestinal system: A species comparison. *Birth Defects Res (Pt B)*, 74:132–156.
27. van den Anker, J.N., Schwab, M., and Kearns, G.L. (2011) Developmental pharmacokinetics. In: Seyberth, H.W. et al., eds., *Handbook of Experimental Pharmacology Pediatric Clinical Pharmacology*. Berlin, Germany: Springer-Verlag.
28. Bartelink, I.H., Rademaker, C.M., Schobben, A.F., and van den Anker, J.N. (2006) Guidelines on paediatric dosing on the basis of developmental physiology and pharmacokinetic considerations. *Clin Pharmacokinet*, 45:1077–1097.
29. Johnson, T.N., Rostami-Hodjegan, A., and Tucker, G.T. (2006) Prediction of the clearance of eleven drugs and associated variability in neonates, infants and children. *Clin Pharmacokinet*, 45:931–956.
30. Kearns, G.L., Abdel-Rahman, S.M., Alander, S.W., Blowey, D.L., Leeder, J.S., and Kauffman, R.E. (2003) Developmental pharmacology—Drug disposition, action, and therapy in infants and children. *N Engl J Med*, 349:1157–1167.
31. Van den Anker, J.N. and Rakhmanina, N.Y. (2006) Pharmacological research in pediatrics: From neonates to adolescents. *Adv Drug Deliv Rev*, 58:4–14.
32. Edgington, A.N., Schmitt, W., Voith, B., and Willmann, S. (2006) A mechanistic approach for the scaling of clearance in children. *Clin Pharmacokinet*, 45:683–704.
33. Anderson, B.J. and Holford, N.H. (2008) Mechanism-based concepts of size and maturity in pharmacokinetics. *Annu Rev Pharmacol Toxicol*, 48:303–332.
34. Blake, M.J., Abdel-Rahman, S.M., Pearce, R.E., Leeder, J.S. and Kearns, G.L. (2006) Effect of diet on the development of drug metabolism by cytochrome P-450 enzymes in healthy infants. *Pediatr Res*, 60:717–723.
35. Van den Anker, J.N., Hop, W., de Groot, R., van der Heijden, A.J., Broerse, H.M. et al. (1994) Effects of pre-natal exposure to betamethasone and indomethacin on the glomerular filtration rate in the preterm infant. *Pediatr Res*, 36:578–581.
36. Allegaert, K., van Schaik, R.H., Vermeersch, S., Verbesselt, R., Cossey, V. et al. (2008) Postmenstrual age and CYP2D6 polymorphisms determine tramadol O-demethylation in critically ill neonates and infants. *Pediatr Res*, 63:674–679.
37. Leeder, J.S. (2003) Developmental and pediatric pharmacogenomics. *Pharmacogenomics*, 4:331–341.
38. Leeder, J.S., Kearns, G.L., Spielberg, S.P., and van den Anker, J.N. (2010) Understanding the relative roles of pharmacogenetics and ontogeny in pediatric drug development and regulatory science. *J Clin Pharmacol*, 50(12):1377–1387.
39. Krekels, E.H., van den Anker, J.N., Baiardi, P., Cella, M., Cheng, K.Y. et al. (2007) Pharmacogenetics and paediatric drug development: Issues and consequences to labelling and dosing recommendations. *Exp Opin Pharmacother*, 8:1787–1799.
40. Kearns, G.L. (2000) Impact of developmental pharmacology on pediatric study design: Overcoming the challenges. *J Allergy Clin Immunol*, 106:S128–S139.
41. Radde, I.C. and McKercher, H.G. (1985) Transport through membranes and development of membrane transport. In: MacLeod, S.M. and Radde, I.C., eds., *Textbook of Pediatric Clinical Pharmacology*. Littleton, MA: PSG, pp. 1–16.
42. Armstrong, R.W., Eichner, E.R., Klein, D.E., Barthel, W.F., Bennett, J.V. et al. (1969) Pentachlorophenol poisoning in a nursery for newborn infants. II. Epidemiologic and toxicologic studies. *J Pediatr*, 75:317–325.
43. Feinblatt, B.I., Aceto, T., Beckhorn, G., and Bruck, E. (1966) Percutaneous absorption of hydrocortisone in children. *Am J Dis Child*, 112:218–224.
44. Evans, W.E., Relling, M.V., Petros, W.P., Meyer, W.H., Mirro, J., Jr., and Crom, W.R. (1989) Dextromethorphan and caffeine as probes for simultaneous determination of debrisoquin-oxidation and N-acetylation phenotypes in children. *Clin Pharmacol Ther*, 45:568–573.
45. Friis-Hansen, B. (1983) Water distribution in the foetus and newborn infant. *Acta Paediatr Scand*, 305:7–11.
46. Kearns, G.L., Jungbluth, G.L., Abdel-Rahman, S.M., Hopkins, N.K., Welshman, I.R. et al. (2003) Impact of ontogeny on linezolid disposition in neonates and infants. *Clin Pharmacol Ther*, 74:413–422.
47. De Hoog, M., Mouton, J.W., and van den Anker, J.N. (2005) New dosing strategies for antibacterial agents in the neonate. *Semin Fetal Neonatal Med*, 10:185–194.
48. Allegaert, K., Anderson, B.J., Verbesselt, R., Debeer, A., de Hoon, J. et al. (2005) Tramadol disposition in the very young: An attempt to assess *in vivo* cytochrome P-450 activity. *Br J Anaesth*, 95:231–239.
49. Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R. et al. (1996) P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6:1–42.
50. Brown, C.M., Reisfeld, B., and Mayeno, A.N. (2008) Cytochromes P450: A structure-based summary of biotransformations using representative substrates. *Drug Metab Rev*, 40:169–184.
51. Hines, R.N. and McCarver, D.G. (2002) The ontogeny of human drug-metabolizing enzymes: Phase I oxidative enzymes. *J Pharmacol Exp Ther*, 300:355–360.
52. McCarver, D.G. and Hines, R.N. (2002) The ontogeny of human drug metabolizing enzymes: Phase II conjugation enzymes and regulatory mechanisms. *J Pharmacol Exp Ther*, 300:361–366.
53. De Wildt, S.N., Kearns, G.L., Leeder, J.S., and van den Anker, J.N. (1999) Glucuronidation in humans: Pharmacogenetic and developmental aspects. *Clin Pharmacokinet*, 36:439–452.
54. Alcorn, J. and McNamara, P.J. (2002) Ontogeny of hepatic and renal systemic clearance pathways in infants: Part I. *Clin Pharmacokinet*, 41:959–998.
55. Hines, R.N. (2008) The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacol Ther*, 118:250–267.
56. Balistreri, W., Zimmer, L., Suchy, F.J., and Bove, K.E. (1984) Bile salt sulfotransferase: Alterations during maturation and non-inducibility during substrate ingestion. *J Lipid Res*, 25:228–235.

57. Card, S.E., Tompkins, S.F., and Brien, J.F. (1989) Ontogeny of the activity of alcohol dehydrogenase and aldehyde dehydrogenases in the liver and placenta of the guinea pig. *Biochem Pharmacol*, 38:2535–2541.
58. Blake, M.J., Castro, L., Leeder, J.S., and Kearns, G.L. (2005) Ontogeny of drug metabolizing enzymes in the neonate. *Semin Fetal Neonatal Med*, 10:123–128.
59. Blake, M.J., Gaedigk, A., Pearce, R.E., Bomgaars, L.R., Christensen, M.L. et al. (2007) Ontogeny of dextromethorphan O- and N-demethylation in the first year of life. *Clin Pharmacol Ther*, 81:510–516.
60. De Wildt, S.N., Kearns, G.L., Hop, W.C., Murry, D.J., Abdel-Rahman, S.M., and van den Anker, J.N. (2001) Pharmacokinetics and metabolism of intravenous midazolam in preterm infants. *Clin Pharmacol Ther*, 70:525–531.
61. Kinirons, M.T., O'Shea, D., Kim, R.B., Groopman, J.D., Thummel, K.E. et al. (1999) Failure of erythromycin breath test to correlate with midazolam clearance as a probe of cytochrome P4503A. *Clin Pharmacol Ther*, 66:224–231.
62. Payne, K., Mattheyse, F.J., Liedenbergh, D., and Dawes, T. (1989) The pharmacokinetics of midazolam in paediatric patients. *Eur J Clin Pharmacol*, 37:267–272.
63. Kerr, B.M., Thummel, K.E., Wurden, C.J., Klein, S.M., Kroetz, D.L. et al. (1994) Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochem Pharmacol*, 47:1969–1979.
64. Pynnonen, S., Sillanpaa, M., Frey, H., and Iisalo, E. (1977) Carbamazepine and its 10,11-epoxide in children and adults with epilepsy. *Eur J Clin Pharmacol*, 11:129–133.
65. Riva, R., Contin, M., Albani, F., Perucca, E., Procaccianti, G., and Baruzzi, A. (1985) Free concentration of carbamazepine and carbamazepine-10,11-epoxide in children and adults. Influence of age and phenobarbitone co-medication. *Clin Pharmacokinet*, 10:524–531.
66. Rane, A., Bertilsson, L., and Palmer, L. (1975) Disposition of placentally transferred carbamazepine (Tegretol) in the newborn. *Eur J Clin Pharmacol*, 8:283–284.
67. Bajpai, M., Roskos, L.K., Shen, D.D., and Levy, R.H. (1996) Roles of cytochrome P450 2C9 and cytochrome P450 2C19 in the stereoselective metabolism of phenytoin to its major metabolite. *Drug Metab Dispos*, 24:1401–1403.
68. Loughnan, P.M., Greenwald, A., Purton, W.W., Aranda, J.V., Watters, G., and Neims, A.H. (1977) Pharmacokinetic observations of phenytoin disposition in the newborn and young infant. *Arch Dis Child*, 52:302–309.
69. Aranda, J.V., Collinge, J.M., Zinman, R., and Watters, G. (1979) Maturation of caffeine elimination in infancy. *Arch Dis Child*, 54:946–949.
70. Kraus, D.M., Fischer, J.H., Reitz, S.J., Kecskes, S.A., Yeh, T.F. et al. (1993) Alterations in theophylline metabolism during the first year of life. *Clin Pharmacol Ther*, 54:351–359.
71. Milavetz, G., Vaughan, L.M., Weinberger, M.M., and Hendeles, L. (1986) Evaluation of a scheme for establishing and maintaining dosage of theophylline in ambulatory patients with chronic asthma. *J Pediatr*, 109:351–354.
72. Rhodin, M.M., Anderson, B.J., Peters, A.M., Coulthard, M.G., Wilkins, B. et al. (2009) Human renal function maturation: A quantitative description using weight and postmenstrual age. *Pediatr Nephrol*, 24:67–76.
73. Chen, N., Aleksa, K., Woodland, C., Rieder, M., and Koren, G.L. (2006) Ontogeny of drug elimination by the human kidney. *Pediatr Nephrol*, 21:160–168.
74. Filler, G. and Lepage, N. (2003) Should the Schwartz formula for estimation of GFR be replaced by cystatin C formula? *Pediatr Nephrol*, 18:981–985.
75. Capparelli, E.V., Lane, J.R., Romanowski, G.L., McFeely, E.J., Murray, W. et al. (2001) The influences of renal function and maturation on vancomycin elimination in newborns and infants. *J Clin Pharmacol*, 41:927–934.
76. Van den Anker, J.N., de Groot, R., Broerse, H.M., Sauer, P.J., van der Heijden, B.J. et al. (1995) Assessment of glomerular filtration rate in preterm infants by serum creatinine: Comparison with inulin clearance. *Pediatrics*, 96:1156–1158.
77. Hayton, W.L. (2002) Maturation and growth of renal function: Dosing renally cleared drugs in children. *AAPS Pharm Sci*, 2:e3.
78. Van den Anker, J.N., Schoemaker, R., Hop, W., van der Heijden, B.J., Weber, A. et al. (1995a) Ceftazidime pharmacokinetics in preterm infants: Effects of renal function and gestational age. *Clin Pharmacol Ther*, 58:650–659.
79. James, L.P., Marotti, T., Stowe, C., Farrar, H.C., Taylor, B., and Kearns, G.L. (1998) Pharmacokinetics and pharmacodynamics of famotidine in infants. *J Clin Pharmacol*, 38:1089–1095.
80. De Hoog, M., Mouton, J.W., Schoemaker, R.C., Verduin, C.M., and van den Anker, J.N. (2002) Extended interval dosing of tobramycin in neonates: Implications for therapeutic drug monitoring. *Clin Pharmacol Ther*, 71:349–358.
81. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies (S3A), Current Step 4 version dated October 27, 1994. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S3A/Step4/S3A_Guideline.pdf
82. Watson, R.E., DeSesso, J.M., Hurtt, M.E., and Cappon, G.D. (2006) Postnatal growth and morphological development of the brain: A species comparison. *Birth Defects Res (Pt B)*, 77:471–484.
83. Dobbing, J. and Sands, J. (1979) Comparative aspects of the brain growth spurt. *Early Hum Dev*, 3:79–83.
84. Haberny, K.A., Paule, M.G., Scallet, A.C., Sistare, F.D., Lester, D.S., Hanig, J.P., and Slikker, W. (2002) Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. *Toxicol Sci*, 68:9–17.
85. Levitt, P. (2003) Structural and functional maturation of the developing primate brain. *J Pediatr*, 143:S35–S45.
86. Klass, P.E., Needleman, R., and Zuckerman, B. (2003) The developing brain and early learning. *Arch Dis Child*, 88:651–654.
87. Noell, N.W. (1955) Metabolic injuries of the visual cell. *Am J Ophthalmol*, 40:60–70.
88. Hussain, N., Clive, J., and Bhandari, V. (1999) Current incidence of retinopathy of prematurity 1989–1997. *Pediatrics*, 104:e26.
89. Dobbing, J. and Sands, J. (1971) Vulnerability of developing brain. IX. The effect of nutritional growth retardation on the timing of the brain growth spurt. *Biol Neonate*, 19:363–378.
90. Wiggins, R.C. (1982) Myelin development and nutritional insufficiency. *Brain Res Rev*, 4:151–175.
91. Romijn, H.J., Hofman, M.A., and Gramsbergen, A. (1991) At what age is the developing cerebral cortex of the rat comparable to that of the full term newborn human baby? *Early Hum Dev*, 26:61–67.
92. Englehardt, B. (2003) Development of the blood–brain barrier. *Cell Tissue Res*, 314:119–129.

93. Morford, L.M., Bowman, C.J., Blanset, D.L., Bogh, I.B., Chellman, G.J., Halpern, W.G., Weinbauer, G.F., and Coogan, T.P. (2011) Preclinical safety evaluations supporting pediatric drug development with biopharmaceuticals: Strategy, challenges, current practices. *Birth Defects Res (Pt B)*, 92:359–380.
94. Virgintino, D., Errede, M., Robertson, D., Capobianco, C., Girolamo, F., Vimercati, A., Bertossi, M., and Roncali, L. (2004) Immunolocalization of tight junction proteins in the adult and developing human brain. *Histochem Cell Biol*, 122:51–59.
95. Cucullo, L. (2009) Prenatal development of the human blood–brain barrier. In: Janigro, D., ed., *Mammalian Brain Development, Contemporary Neuroscience*. New York: Humana Press, pp. 53–75.
96. Englehardt, H.H. and Valyi-Nagy, T. (2005) Biology of the blood–brain and “blood–brain tumor” barriers. In: Newton, H., ed., *Handbook of Brain Tumor Chemotherapy*. San Diego, CA: Elsevier, pp. 236–243.
97. Pardridge, W.M. (2001) BBB-genomics: Creating new openings for brain–drug targeting. *Drug Discov Today*, 6:381–383 as reprinted in Pardridge, W.M. (2003) Blood–brain barrier drug targeting: The future of brain drug development. *Mol Interventions*, 3(2): <http://melanotan.org/yabbfiles/Attachments/BBB.PDF>
98. Scheuplein, R., Charnley, G., and Dourson, M. (2002) Differential sensitivity of children and adults to chemical toxicity: I. Biological basis. *Regul Toxicol Pharmacol*, 35(3):429–447.
99. Costa, L.G., Aschner, M., Vitalone, A., Syversen, T., and Soldin, O.P. (2004) Developmental neuropathology of environmental agents. *Annu Rev Pharmacol Toxicol*, 44:87–110.
100. Bauer, H.-C. and Bauer, H. (2000) Neural induction of the blood–brain barrier: Still an enigma. *Cell Mol Neurobiol*, 20:13–28.
101. Wood, S.L., Beyer, B.K., and Cappon, G.D. (2003) Species comparison of postnatal CNS development: Functional measures. *Birth Defects Res (Pt B)*, 68:391–407.
102. Alberts, J.A. (1984) Sensory-perceptual development in the Norway rat: A view toward comparative studies. In: Kail, R. and Spear, N.E., eds., *Comparative Perspectives on the Development of Memory*. Hillsdale, NJ: Erlbaum, pp. 65–101.
103. Richardson, R., Paxinos, G., and Lee, J. (2000) The ontogeny of conditioned odor potentiation of startle. *Behav Neurosci*, 114:1167–1173.
104. Teicher, M.H. and Blass, E.M. (1977) First suckling response of the newborn albino rat: The roles of olfaction and amniotic fluid. *Science*, 196:635–636.
105. Hudson, R. and Distel, H. (1983) Nipple location by newborn rabbits: Behavioral evidence for pheromonal guidance. *Behavior*, 85:260–275.
106. Yamaguchi, K., Harada, S., Kanemaru, N., and Kasahara, Y. (2001) Age-related alteration of taste bud distribution in the common marmoset. *Chem Senses*, 26:1–6.
107. Stickord, G., Kimble, D.P., and Smotherman, W.P. (1982) *In utero* taste/odor aversion conditioning in the rat. *Physiol Behav*, 28:5–7.
108. Pedersen, P.E., Stewart, W.B., Green, A.G., and Shepherd, G.M. (1983) Evidence of olfactory function *in utero*. *Science*, 221:478–480.
109. Vogt, M.B. and Rudy, J.W. (1984) Ontogenesis of learning: IV. Dissociation of memory and perceptual-altering processes mediating taste neophobia in the rat. *Dev Psychobiol*, 17:601–611.
110. Girden, E., Mettler, F.A., Finch, G., and Culler, E. (1936) Conditioned responses in a decorticate dog to acoustic, thermal, and tactile stimulation. *J Comp Psychol*, 21:367–385.
111. LeDoux, J.E., Sakaguchi, A., and Reis, D.J. (1984) Subcortical efferent projections of the medial geniculate nucleus mediate emotional responses conditioned to acoustic stimuli. *J Neurosci Methods*, 4:383–398.
112. Adams, J. (1986) Methods of behavioral teratology. In: Riley, E.P. and Vorhees, C.V., eds., *Handbook of Behavioral Teratology*. New York: Plenum Press, pp. 67–97.
113. U.S. FDA (CDER) (CBER) ICH E11 Guidance for industry: Nonclinical safety evaluation of pediatric drug products, December 2000. <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129477.pdf>
114. Hew, K.W. and Keller, K.A. (2003) Postnatal anatomical and functional development of the heart: A species comparison. *Birth Defects Res (Pt B)*, 68:309–320.
115. Henning, S.J. (1981) Postnatal development: Coordination of feeding, digestion, and metabolism. *Am J Physiol*, 241:G199–G214.
116. Buddington, R.K. (1994) Nutrition and ontogenetic development of the intestine. *Can J Physiol Pharmacol*, 72:251–259.
117. Pacha, J. (2000) Development of intestinal transport function in mammals. *Physiol Rev*, 80:1633–1667.
118. de Zwart, L., Scholten, M., Monbaliu, J.G., Annaert, P.P., Van Houdt, J.S. et al. (2008) The ontogeny of drug metabolizing enzymes and transporters in the rat. *Reprod Toxicol*, 26:220–230.
119. Holsapple, M.P., West, L.J., and Landreth, K.S. (2003) Species comparison of anatomical and functional immune system development. *Birth Defects Res (Pt B)*, 68:321–334.
120. Weissman, I. (2000) Stem cells: Units of development, units of regeneration and units in evolution. *Cell*, 100:157–168.
121. Good, R.A. (1995) Organization and development of the immune system. Relation to its reconstruction. *NY Acad Sci*, 77:8–33.
122. Migliaccio, G., Migliaccio, A.R., Petti, S., Mavilio, F., Russo, G. et al. (1986) Human embryonic hemopoiesis: Kinetics of progenitors and precursors underlying the yolk sac–liver transition. *J Clin Invest*, 78:51–60.
123. Haynes, B.F., Martin, M.E., Kay, H.H., and Kurtzberg, J. (1988) Early events in human T cell ontogeny. *J Exp Med*, 168:1061–1080.
124. Cumano, A. and Godin, I. (2001) Pluripotent hematopoietic stem cell development during embryogenesis. *Curr Opin Immunol*, 13:166–171.
125. Barnett, J.B. (1996) Developmental immunotoxicology. In: Smialowicz, R.J. and Holsapple, M.P., eds., *Experimental Immunotoxicology*. Boca Raton, FL: CRC Press, pp. 47–62.
126. Dietert, R.R., Etzel, R.A., Chen, D., Halonen, M., Holladay, S.D., Jarabek, A.M., Landreth, K., Peden, D.B., Pinkerton, K., Smialowicz, R.J., and Zoetis, T. (2000) Workshop to identify critical windows of exposure for children’s health: Immune and Respiratory Systems Work Group summary. *Environ Health Perspect*, 108(Suppl):483–490.
127. Zoetis, T. and Hurtt, M.E. (2003) Species comparison of anatomical and functional renal development. *Birth Defects Res (Pt B)*, 68:111–120.
128. Bernstein, J. (1991) Morphologic development and anatomy. In: Rudolph, A.M., ed., *Rudolph’s Pediatrics. The Kidneys and Urinary Tract*. Norwalk, CT: Appleton & Lange, pp. 1223–1224.
129. Marty, M.E., Chapin, R.E., Parks, L.G., and Thorsrud, B.A. (2003) Development and maturation of the male reproductive system. *Birth Defects Res (Pt B)*, 68:125–136.

130. Beckman, D.A. and Feuston, M. (2003) Landmarks in the development of the female reproductive system. *Birth Defects Res (Pt B)*, 68:137–143.
131. Zoetis, T. and Hurt, M.E. (2003) Species comparison of lung development. *Birth Defects Res (Pt B)*, 68:121–124.
132. Thurlbeck, W.M. (1975) Postnatal growth and development of the lung. *Am Rev Respir Dis*, 111:803–844.
133. Lau, C. and Kavlock, R.J. (1994) Functional toxicity in the developing heart, lung, and kidney. In: Kimmel, C.A. and Buelke-Sam, J., eds., *Developmental Toxicology*, 2nd edn., New York: Raven Press, pp. 119–188.
134. Buelke-Sam, J., Kimmel, C.A., Adams, J., Nelson, C.J., Vorhees, C.V. et al. (1985) Collaborative behavioral teratology study: Results. *Neurobehav Toxicol Teratol*, 7(6):591–624.
135. Historical Control Data for Development and Reproductive Toxicity Studies using the CrI:CD® BR Rat. (2013) Compiled by E.M. Lewis. www.criver.com
136. Diehl, K.-H., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.-M., and van de Vorstenbosch, C. (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol*, 21:15–23.
137. Turner, P.V., Brabb, T., Pekow, C., and Vasbinder, M.A. (2011) Administration of substances to laboratory animals: Routes of administration and factors to consider. *J Am Assoc Lab Anim Sci*, 50(5):600–613.
138. Bates, H.K., Cunney, H.C., and Kebede, G.A. (1997) Developmental neurotoxicity testing methodology. In: Hood, R.D., ed., *Handbook of Developmental Toxicology*. Boca Raton, FL: CRC Press, p. 291.
139. Henck, J.W. (2002) Developmental neurotoxicology: Testing and interpretation. In: Massaro, E.J., ed., *Handbook of Neurotoxicology*, Vol. II. Totowa, NJ: Humana Press, p. 461.
140. Iezhitsa, I.N., Spasov, A.A., and Bugaeva, L.I. (2001) Effects of Bromantan on offspring maturation and development of reflexes. *Neurotoxicol Teratol*, 23:213–222.
141. Cummings, A.M. and Gray, L.E. (1987) Methoxychlor affects the decidual cell response of the uterus but not other progestational parameters in female rats. *Toxicol Appl Pharmacol*, 90:330.
142. Lewis, E.M., Christian, M.S., Barnett, J., Jr., and Hoberman, A.M. (2002) Control preputial separation data for F₁ generation CRL Sprague-Dawley (“Gold Standard”) rats in EPA Developmental Neurotoxicology, EPA Multigeneration and FDA Peri-Postnatal Studies. *Toxicologist*, 66(1):1149.
143. Lewis, E.M., Barnett, J.F., Jr., Freshwater, L., Hoberman, A.M., and Christian, M.S. (2002) Sexual maturation data for CRL Sprague-Dawley rats: Criteria and confounding factors. *Drug Chem Toxicol*, 25(4):437.
144. Tinwell, H., Haseman, J., Lefevre, P.A., Wallis, N., and Ashby, J. (2002) Normal sexual development of two strains of rat exposed *in utero* to low doses of bisphenol A. *Toxicol Sci*, 68:339.
145. Hoberman, A.M., Christian, M.S., Lewis, E.M., and Barnett, J., Jr. (2002) Control vaginal opening data for F₁ generation CRL Sprague-Dawley (“Gold Standard”) rats in EPA Developmental Neurotoxicology, EPA Multigeneration and FDA Peri-Postnatal Studies. *Toxicologist*, 66(1):1137.
146. AVMA Guidelines on Euthanasia (Formerly Report of the AVMA Panel on Euthanasia), June 2007, <https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>
147. “When ovarian hysterectomies are performed, euthanasia of feti should be accomplished as soon as possible after removal from the dam. Neonatal animals are relatively resistant to hypoxia”. 2000 Report of the AVMA Panel on Euthanasia. *JAVMA*, 218:688.
148. Phifer, C.B. and Terry, L.M. (1986) Use of hypothermia for general anesthesia in preweanling rodent. *Physiol Behav*, 38:887–890.
149. Insel, P.A. (1996) Analgesic-antipyretic and anti-inflammatory agents. In: Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W., and Gilman, A.G., eds., *Goodman & Gilman’s The Pharmacological Basis of Therapeutics*, 9th edn. New York: McGraw-Hill, p. 632.
150. Dreifuss, F.E., Santilli, N., Langer, D.H., Sweeney, K.P., Moline, K.A. et al. (1987) Valproic acid hepatic fatalities: A retrospective review. *Neurology*, 37:379–385.
151. Kapusnik-Uner, J.E., Sande, M.A., and Chambers, H.F. (1996) Antimicrobial agents. In: Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W., and Gilman, A.G., eds., *Goodman & Gilman’s The Pharmacological Basis of Therapeutics*, 9th edn. New York: McGraw-Hill, pp. 1124–1153.
152. FDA Talk Paper, November 9, 1998, Class labeling for intranasal and orally inhaled corticosteroid containing drug products regarding the potential for growth suppression in children.
153. Belay, E.D., Bresee, J.S., Holman, R.C., Khan, A.S., Shahriari, A. et al. (1999) Reye’s syndrome in the United States from 1981 through 1997. *N Engl J Med*, 340:1377–1382.
154. Guberman, A.H., Besag, F.M., Brodie, M.J., Dooley, J.M., Duchowny, M.S. et al. (1999) Lamotrigine-associated rash: Risk/benefit considerations in adults and children. *Epilepsia*, 40:985–991.
155. Strolin Benedetti, M. and Baltes, E.L. (2003) Drug metabolism and disposition in children. *Fundam Clin Pharmacol*, 17:281–299.
156. Nachman, R.L. and Esterly, N.B. (1980) Increased skin permeability in preterm infants. *J Pediatr*, 96:99–103.
157. Ginsberg, G., Hattis, D., Miller, M., and Sonawane, B. (2004) Pediatric pharmacokinetic data: Implications for environmental risk assessment for children. *Pediatrics*, 113:973–983.
158. Kimmel, C.A. and Buelke-Sam, J. (1994) *Target Organ Toxicology Series*. New York: Taylor & Francis.
159. Kearns, L.K. and Reed, M.D. (1989) Clinical pharmacokinetics in infants and children. A reappraisal. *Clin Pharmacokinet*, 17(Suppl 1):29–67.
160. Leeder, J.S. and Kearns, G.L. (1997) Pharmacogenetics in pediatrics: Implications for practice. *New Front Pediatr Drug Ther, Pediatr Clin N Am*, 44(1):55–77.
161. Waxman, D.J., Morrissey, J.J., and Le Balnc, G.A. (1989) Female predominant rat hepatic P450 forms (IIE1) and 3(IIA1) are under hormonal regulatory controls distinct from those of sex specific P450 forms. *Endocrinology*, 270(N2):458–471.
162. Peng, H.M., Porter, T.D., Ding, X.X., and Coon, M.J. (1991) Differences in the Developmental expression of rabbit cytochromes P450 2E1 and 2E2. *Mol Pharmacol*, 40(N1):58–62.
163. Ding, X., Peng, H.M., and Coon, M.F. (1992) Cytochromes P450 NMa, NMb (2G1) and LM4 (1A2) are differentially expressed during development in rabbit olfactory mucosa and liver. *Mol Pharmacol*, 42(N6):1027–1032.
164. Imaoka, S., Fujita, S., and Funae, Y. (1991) Age dependent expression of cytochrome P450s in rat liver. *Biochem Biophys Acta*, 1097(N3):187–192.
165. Pineau, T., Daujat, M., Pichard, L., Girard, F., Angevain, J. et al. (1991) Developmental expression of rabbit cytochrome P450 *CYP1A1*, *CYP1A2*, *CYP3A6* genes. Effect of weaning and rifampicin. *Curr J Biochem*, 197(N1):145–153.
166. Snodgrass, W.R. (1992) Physiological and biochemical differences between children and adults as determinants of toxic response to environmental pollutants, similarities and differences between children and adults, implications for risk

- assessment. In: *Similarities and Differences Between Children and Adults: Implications for Risk Assessment*, Guzelian, P.S., Henry, C.J., and Olin, S.S., eds. Washington, DC: ILSI Press, pp. 35–42.
167. Travis, L.B. (1991) The Kidney and Urinary Tract Morphogenic Development and Anatomy. In: *Rudolph's Pediatrics*, 19th Edn., Chapter 25, C. Rudolph et al. eds., pp. 1223–1236.
168. Zoetis, T. (2003) Species comparison of anatomical and functional renal development. *Birth Defects Res (Pt B)*, 68:111–120.
169. DeSesso, J.M. and Harris, S.B. (1995) Principles underlying developmental toxicity, toxicology risk assessment. In: *Toxicology and Risk Assessment*, Fan, A.M. and Chang, L.W., eds., New York: Marcel Dekker.
170. Zeltner, T.B., Cauduff, J.H., Gehr, P., Pfenninger, J., and Burri, P.H. (1987) The postnatal development and growth of the human lung. I. *Morphom Respir Physiol*, 67:247–267.
171. Bailey, G. and Schaepdrijver, L.D. (2010) *ILSI/HESI Workshop: The Value of Juvenile Toxicity Studies: Introduction and 'Warm up,'* Washington, DC, April 5–6, unpublished workshop.
172. Hoberman, A.M. and Lewis, E.M., eds. (2010) *Pediatric Non-Clinical Drug Testing: Principles, Requirements and Practices*. Hoboken, NJ: John Wiley & Sons.
173. U.S. Environmental Protection Agency. *Toxic Substances Control Act Testing Guidelines*, 40 CFR Part 798 Subpart G Section 798.6050. Fed Reg. 1985; 50:39458.
174. Laffan, S.B. and Posobiec, L. (2012) Approaches to rat juvenile toxicity studies and case studies: A pharmaceutical perspective. In: Hoberman, A.M. and Lewis, E.M., eds., *Pediatric Non-Clinical Drug Testing*, Chapter 14. Hoboken, NJ: John Wiley & Sons.
175. Kaufman, R.E. (1992) Drug action and therapy in infant and child. In: *Pediatric Pharmacology: Therapeutic principles in practice, 2nd Edn.*, S.J. Yaffe and J.V. Aranda, eds., pp. 212–219. W.B. Saunders Company.
176. Collinge, M., Burns-Naas, L.A., Chellman, G.J., Kawabata, T.T., Komocsar, W.J., Piccotti, J.R., Jacintha Shenton, J., and Wier, D. (2012) Developmental immunotoxicity (DIT) testing of pharmaceuticals: Current practices, state of the science, knowledge gaps, and recommendations. *J Immunotoxicol*, Early Online: 1–21.
177. Buelke-Sam, J. (2011) Comparative age categories in juvenile toxicity studies, Presentation at the *FDA/CDER Office of New Drugs "Juvenile Animal Study Workshop,"* Silver Spring, MD, May 2011.
178. Lewis, Z.M., De Schaepdrijver, L.M. and Coogan, T.P. (2012) In: Hoberman, A.M. and Lewis, E.M., eds., *Pediatric Non-Clinical Drug Testing*. Chapter 14, p.26. Hoboken, NJ: John Wiley & Sons.

12 Endocrine Toxicology

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INTRODUCTION

The evolutionary leap to multicellular animals enabled huge increases in organismic size, structural and functional specialization, and complexity of cells into tissues, organs, and organ systems. However, it also required development of accurate (usually rapid) communication among these disparate cells, tissues, and organs. Three such communication systems evolved:

1. The nervous system (with the brain as the coordinating and communications center) that provides very rapid transfer of information from sensory organs and response information to effector organs, via afferent and efferent neuronal pathways, respectively, to provide rapid responses
2. The circulatory system (with the heart as the central pump and the arteries, capillaries, and veins as distribution conduits) that provides O₂/CO₂ exchange, nutrient/waste exchange, and delivery of signaling molecules such as hormones, etc.
3. The endocrine system (with the pituitary gland, just under the brain, as the coordinating center between the brain and the other endocrine glands), an intercellular system of specialized organs that responds to external or internal changes (by means of the nervous system) by production of hormones from various glands (i.e., chemical communication) which are delivered to various target structures/organs (by means of the circulatory system). These hormones cause internal responses/adjustments to environmental changes detected (e.g., day length, ambient temperature, food quality and/or quantity, predators, etc.), or to internal changes, during pre- and postnatal development and maturation of pubertal and adult reproductive structures and functions in males and females. These structures and/or functions include changes in sex-specific internal and external features, such as male and female reproductive organs, plumage or pelage coloring, and behaviors such as mating

cries, songs, dances, bower building, etc. that result in production of eggs/sperm, partner bonding, nesting, mating, and raising of young.

There are three types of chemical communication systems which evolved: within the cell (via intracellular fluid, termed autocrine), cell to cell (via intercellular fluid, termed paracrine), and over distances within a multicellular organism (via hormones released into the circulatory system) or between such organisms (e.g., for nesting, mating, or maternal–offspring bonding, etc. via pheromones, released into the environment), termed the endocrine system. For multicellular organisms, there is an absolute necessity for such an intercellular system to communicate, coordinate, and control recognition of internal and external cues and changes, and to communicate, coordinate, and control appropriate timely responses as well as control of growth, sexual maturity, reproduction, osmoregulation, stress, and metabolism [1], that is, an endocrine system.

This chapter is organized into the following three major sections:

1. Embryological origin, development, and functions of the vertebrate endocrine system
2. Evolution of the vertebrate endocrine system
3. Endocrine toxicity and national and international regulatory activities

EMBRYOLOGICAL ORIGIN, DEVELOPMENT, AND FUNCTIONS OF THE VERTEBRATE ENDOCRINE SYSTEM

BACKGROUND

The vertebrate endocrine system is located within specific endocrine organs and in organs and tissues with other specific functions. The endocrine glands produce hormones, distributed by the circulatory (vascular) system to the various hormone-sensitive tissues, which are highly vascularized. There are three types of hormones:

1. Amino acid derivatives, example, noradrenaline (norepinephrine), adrenaline (epinephrine), and thyroid hormone (TH)
2. Proteins/peptides: Thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH)
3. Steroids, example, androgens, estrogens, glucocorticoids, and mineralocorticoids

Circulating hormones are recognized by their target hormone-responsive cells using cell surface receptors for modified amino acids, peptides, and proteins, or by cytoplasmic/nuclear receptors for steroids. Hormonal effects can be rapid or slow, brief or prolonged, diurnal or continuous, short or long term. Hormone effects can also be mimicked, stimulated, or blocked by therapeutic drugs, “natural” nutritional chemicals, and natural and manmade environmental chemicals, acting as endocrine agonists or antagonists.

Various explanations have been proposed to explain the complex differentiation and development in higher vertebrates including humans, since the human genome contains only two to three times the numbers of genes as invertebrates. It is highly likely that the activities of the various endocrine glands (e.g., adrenal, pituitary and gonadal sex steroids such as androgens, estrogens, glucocorticoids, mineralocorticoids, and progestins) acting through receptors (which arose early on from a primitive nuclear receptor in primitive protochordates) provide the additional complexity in vertebrate development and differentiation [2]. In fact, hormones are considered mediators of development [3].

A major group of hormones are the sex steroids, so named for their effects on sexually dimorphic development of the reproductive tract, secondary sex characteristics, and central nervous system, which result in sexually dimorphic behavior and physiology. Receptors for sex steroids are ubiquitous. Sex steroids are synthesized *de novo* from cholesterol in the brain (in the case of brain neurosteroids), and in the gonads and adrenal glands. Sex steroids may also be generated by conversion from other (mostly adrenal) circulating steroids. These sex steroids are released into the blood supply for distribution [4].

The onset and process of puberty, one of the most critical and sensitive stages in reproductive development, is controlled by the neuroendocrine system. Recent studies have reported that the age of the onset of puberty has shifted to earlier ages by 1–2 years in children, with no change in menarche, and also in domestic and wild animals. The triggers for onset of puberty are genetic, hormonal/endocrine, and environmental, and these triggers are therefore also likely to be the causes of the decreased ages at puberty onset. A number of natural and synthetic endocrine-disrupting chemicals have been implicated in the acceleration of puberty onset worldwide, such as phytoestrogens, diethylstilbestrol (DES), pesticides, fungicides, herbicides, cleaning substances, components of cosmetic products, dyes, plastics, solvents, etc. [5].

The vertebrate endocrine system currently consists of eight major individual or paired glands:

1. Pituitary (or hypophysis) with two lobes, the adenohypophysis (anterior lobe) and the neurohypophysis (posterior lobe), located just beneath the brain, with direct communication to and from the brain via the hypothalamus (part of the brain), and to the other glands by its release of regulatory hormones (stimulatory and inhibitory) into the circulatory system
- The other glands, under appropriate control, in turn, release their hormones into the circulatory system for distribution. The other glands include
2. Pineal gland
3. Thymus
4. Thyroid gland
5. Parathyroid gland located in the neck region
6. Pancreas (islets of Langerhans) located under the stomach
7. Paired adrenal glands located just above and medial to the kidneys

8. Paired gonads, ovaries in females, testes in males, located in the lower abdomen below the kidneys, which produce not only the reproductive cells (eggs and sperm) released into the water for external union (fertilization) in most aquatic vertebrates, or just the sperm introduced into the female (where the eggs are fertilized internally in some aquatic and all land vertebrates), but also the reproductive hormones, which are released into the circulatory system, and pheromones released into the environment

Other endocrine sources in organs with other specific functions include the endocrine heart, the endocrine kidney, the endocrine gastrointestinal tract (GIT), and the adipose tissue. In addition, during in utero development in mammals, there is the temporary placenta, producing hormones for the conceptus, and transporting hormones (among other things) made by the mother to the conceptus via the placenta [6]. See Table 12.1 for a list of hormones, their sources, and functions.

TABLE 12.1
Vertebrate Endocrine System

Gland	Hormone(s)	Major Effect(s)
Hypothalamus (part of brain)	Thyrotropin-releasing hormone Corticotropin-releasing hormone Arginine vasopressin Gonadotropin-releasing hormone Somatostatin Prolactin-releasing hormone Dopamine Aromatase Growth hormone-releasing hormone	Controls the thyroid gland Controls the adrenal glands Controls the kidneys (antidiuretic) Controls the gonads and steroid hormone release Inhibits growth hormone Releases prolactin from anterior pituitary cells Functions as a neurotransmitter Converts testosterone to estrogen Regulates release of growth hormone
Pineal gland	Melatonin	Regulates biorhythms, influences reproduction in some species
Pituitary gland	Thyroid-stimulating hormone Adrenocorticotrophic hormone Luteinizing hormone Follicle-stimulating hormone Somatotrophin (growth hormone) Prolactin Melanocyte-stimulating hormone Oxytocin Arginine vasopressin Activin Inhibin Secretoneurin	Stimulates secretion of thyroid hormone Stimulates secretion of adrenal cortex hormones Stimulates ovulation and production of estrogen, progesterone, and testosterone Stimulates production of ova and sperm Promotes growth Stimulates milk production and maintains estrogen and progesterone secretion Stimulates dispersal of pigment Stimulates contraction of uterus and secretion of milk Regulates water retention, reabsorption of molecules in kidney tubules; increases arterial blood pressure Stimulates FSH synthesis/secretion Downregulates FSH Stimulates LH production
Thyroid gland	Thyroid hormone	Stimulates and maintains metabolism
Parathyroid gland	Parathyroid hormone	Raises blood calcium
Thymus	Thymosin	Stimulates T-lymphocytes
Pancreas	Glucagon Insulin Somatostatin Pancreatic polypeptide	Raises blood glucose Lowers blood glucose Inhibits growth hormone Regulates pancreatic activities, has effects on hepatic glycogen levels
Adrenal gland (cortex)	Dehydroepiandrosterone Cortisol Aldosterone	Metabolic intermediate in the biosynthesis of androgen and estrogen Raises blood glucose, suppresses the immune system Affects the kidney, conserving sodium, secreting potassium, increasing water retention, and raising blood pressure
Gonads		
Testes	Testosterone Androstenedione	Supports spermatogenesis, promotes development and maintenance of secondary sex characteristics Intermediate in synthetic pathway for testosterone, estrogen, and estradiol (also made in the adrenal gland)
Ovaries	17 β -Estradiol Estriol Estrone Estrogen Progesterone	Stimulates growth of uterine lining, promotes development and maintenance of secondary sex characteristics Made during pregnancy, is weakly estrogenic Produced from androstenedione, reservoir for estradiol (also made in adipose tissue) Important to estrous cycle. Primary female sex hormone Promotes growth of uterine lining

DEVELOPMENT AND FUNCTIONS OF THE MAJOR VERTEBRATE ENDOCRINE GLANDS

The *hypothalamus*, part of the brain [7], develops from the neuroectoderm after formation of the early prosencephalon (the forebrain) which becomes the diencephalon. It is located just below the thalamus, and just above the brain stem, forming the ventral part of the diencephalon in all vertebrate brains. The cells of the ventrolateral wall in the intermediate zone proliferate, and form mammillary bodies (pea-sized swellings in the ventral wall of the hypothalamus) which control feeding reflexes and neurohormones, body temperature, thirst, and circadian rhythms. The hypothalamus is a link from the nervous system to the endocrine system via the pituitary gland (the hypophysis). It is responsible for some metabolic processes, some activities of the autonomic nervous system, and secretes certain neurohormones (hypothalamic-releasing hormones) which stimulate or inhibit pituitary hormone secretions. It controls body temperature, hunger, thirst, fatigue, and circadian rhythms. The hypothalamic-releasing hormones include the thyrotrophin-releasing hormone (TRH) which affects/controls the thyroid gland, corticotrophin-releasing hormone (CRH) which affects/controls the adrenal glands, arginine vasopressin (AVP), the antidiuretic hormone which affects/controls the kidneys, gonadotrophin-releasing hormone (GnRH) which affects/controls the gonads and steroid release in vertebrates, growth hormone-releasing hormone (GHRH), somatostatin, which inhibits growth hormone (GH), dopamine, and activin and inhibin [8]. A prolactin-releasing peptide or factor (PRF) has also been found in the hypothalamus [9].

GnRH is considered the head of the hypothalamic-pituitary-gonadal (HPG) regulatory cascade, and is highly conserved among vertebrates. It is referred to as the "Master Molecule of Reproduction." Hypothalamic GnRH neurons are affected by over 30 neurotransmitters, neuropeptides, cytokines, hormones, and growth factors in the brain [4]. The hypothalamus also produces aromatase (which converts testosterone to estrogen) as do the gonads, both of which can be affected by temperature and estrogen exposure, causing reproductive anomalies in embryonic alligators [10] and affected by endocrine-active environmental contaminants, as in paper mill effluents causing comparable reproductive anomalies in the Eastern mosquito fish [11].

The *pineal gland* is part of the epithalamus, formed from the neuroectoderm after the early prosencephalon (forebrain) develops into the diencephalon, on the caudal roof as a median diverticulum. Initially, the diverticulum is hollow; cell proliferation fills the gland which converts it into a solid body. It receives sympathetic, parasympathetic, and trigeminal ganglionic innervation. It forms neurons and pinealocytes (neuroglia); pinealocytes secrete melatonin which is released cyclically, stimulated by darkness, and inhibited during daylight. The abundant melatonin in children inhibits hypothalamic secretion of GnRH and therefore inhibits sexual development until puberty, when melatonin production is reduced, allowing pubertal sexual development. Other activities

of the pineal gland may include effects on gamete maturation, and antioxidant effects to protect neurons.

The *pituitary gland* is bilobed, consisting of the anterior adenohypophysis and the posterior neurohypophysis. It has dual ectodermal origins: the ectodermal roof of the stomodaeum forms Rathke's pouch and the adenohypophysis, while the neuroectoderm from the early prosencephalon, which differentiates into the diencephalon, forms the neurohypophysis. The anterior wall of the adenohypophysis proliferates to form the pars distalis; the posterior wall exhibits little growth and forms the pars intermedia (PA); and the rostral growth around the infundibular stem forms the pars tuberalis. The anterior pituitary produces TSH, adrenocorticotrophic hormone (ACTH), LH, FSH, somatotrophin/GH, prolactin (PRL), and melanocyte-stimulating hormone (MSH). The posterior pituitary produces oxytocin and AVP. Brain regions have estrogen receptors (ERs), as do glands and most other structures.

Activin and inhibin are two closely related protein dimers with opposite biological effects; both have identical/similar β -subunits, with differing α -subunits. Both activin and inhibin are produced in the pituitary gland, the gonads, and other organs, including the bone marrow and spleen [12] and the hypothalamus (see earlier [8]), with activin also produced in the placenta and the ovarian corpora lutea. They both modulate human monocyte chemotaxis and human lymphocyte interferon- γ production [12].

Activin enhances FSH synthesis/secretion and plays a role in the regulation of the menstrual cycle [8]. In the ovarian follicle, activin increases FSH binding and FSH-induced aromatization (to convert testosterone to estrogen). It also participates in androgen synthesis by enhancing LH activity in both male and female gonads. In males, it enhances spermatogenesis. It regulates the morphogenesis of branching organs, such as the prostate, lungs, and kidneys. It also increases the expression level of type 1 collagen, so it is a likely a potent activator of fibroblasts. It also plays a role in cell proliferation, differentiation, apoptosis, metabolism, homeostasis, immune responses, wound repair (accelerating healing and scar formation), and endocrine function. Activins interact with Types I and II cell surface transmembrane receptors, activating type 1 activin receptor which phosphorylates SMAD proteins, which allow expression of many genes [13]. Lack of activin during development results in neurodevelopmental defects [14–16].

Conversely, inhibin downregulates FSH synthesis and inhibits FSH secretion. In females, FSH stimulates the secretion of inhibin from the granulosa cells of the ovarian follicle, which, in turn, suppresses FSH. Inhibin secretion is reduced by GnRH and increased by insulin-like growth factor 1 (IGF-1). In males, inhibin is secreted by the Sertoli cells (in the seminiferous tubules of the testes). Androgens stimulate inhibin production which may then participate in the local regulation of spermatogenesis. Little is known about the inhibin mechanism(s) of action [16,17]). (See the later section on the evolution of the gonads for more information on inhibin.)

Secretoneurin (SN) is a 33–34 amino acid peptide produced in the pituitary gland, derived from the most conserved

sequence of the secretogranin (Sg11) precursor. Sg11 is found in secretory granules of neuroendocrine tissues, with two paralogues in Teleost fishes, Sg11a and Sg11b. SN immunoreactivity is found in all major pituitary cell types in mammals. In goldfish, it appears to be mainly expressed in the PRL cells of the rostral pars distalis (RPD). Goldfish SN stimulates LH in the gonadotrophs of the proximal pars distalis. SN is also produced by hypophysiotropic neurons which deliver SN to target cells by direct pituitary innervation. The PRL secretagogue, TRH, stimulates secretion of mature Sg11 and its derived peptides in Sg11-producing cells in the pituitary, when treated with 17β -estradiol, epidermal growth factor, and insulin; increased cell content of Sg11 is concordant with PRL expression. Little is known about SN's neuroendocrine function(s), although it is known to play an essential role in immune and inflammatory responses. Zhao et al. [18] have shown that SN stimulates LH production and release in the goldfish pituitary by manipulating calcium ion levels in pituitary gonadotrophs. When an SN receptor and the signaling cascade in gonadotrophs are identified, then the authors "will be able to propose SN as a new hormone in the neuroendocrine system" [18].

Soy isoflavones bind to ERs in the pituitary gland and can cause in vivo estrogenicity, developmental toxicity, and estrogen-mediated developmental carcinogenesis in rats. Genistein, the major soy isoflavone, has known estrogenic effects and is used medically to treat women with reduced estrogen [19].

The human pituitary developmental time line is as follows: by Week 4, the hypophysial pouch is present, Rathke's pouch begins as a diverticulum from the ectodermal stomadeal roof; by Week 5, the growing pouch elongates and contacts the infundibulum, a diverticulum of the diencephalon; by Week 6, the connecting stalk between the pouch and the oral cavity degenerates; by Week 10, GH and ACTH are first detectable; by Week 16, the adenohypophysis is fully differentiated; and by Weeks 20–24 (still in utero), GH levels peak and then decline.

The *thyroid gland* begins as a median endodermal thickening (out-pouching) in the floor of the pharynx; this out-pouching is the thyroid diverticulum, which is present in all very young vertebrate embryos (including humans). The tongue also grows from the pharyngeal floor and elongates; the thyroid cells then descend, along with the tongue, into the neck region. The thyroglossal duct forms at the end of the foramen cecum of the tongue. Additional lateral contributions to the embryonic thyroid come from the fourth pharyngeal arches, and the neural crest which is the source of the thyroid calcitonin-producing parafollicular cells (C cells). The thyroid diverticulum is at first hollow; it then fills with cells forming right and left lobes and a central isthmus. The thyroid gland tissues merge and migrate to their final location by GD 45–50 in humans. The thyroid gland begins to function at Week 10 (GD 70) in the human embryo, producing TH, which is required for neural development, especially brain development. By Week 11 in the human fetus, colloid appears in the thyroid follicles, as does iodine which initiates TH synthesis. The THs are amino acid derivatives: thyroxine (T4) and triiodothyronine

(T3), both synthesized from iodine atoms and tyrosine. The pre-/perinatal reduction or blockage of production of TH (e.g., from iodine deficiency) leads to neurological defects, example, cretinism (severely stunted physical and mental growth, resulting in mental retardation). But cretinism also has other causes. It has also been shown to be caused by combined reduced pituitary hormone deficiencies of thyrotropin (which controls thyroid activity; see later), GH and PRL, all due to a nonsense mutation in the gene for a pituitary-specific transcription activator, which then affects thyroid gland development, differentiation, and maintenance, including TH production [20]. The thyroid gland also produces calcitonin, which plays a role in calcium homeostasis.

TH also stimulates protein, lipid, and carbohydrate metabolism; reduction in metabolism can also cause cretinism. Growth factors (IGF and epidermal growth factors) stimulate thyroid follicular growth. Fetal TH is secreted into the blood supply; it is biologically inactivated by modification in the liver; late fetal secretion also stimulates the production of brown fat. Fetal self-sufficiency of THs is thought to protect the fetus against effects on brain development from maternal hypothyroidism in most cases. However, preterm births can result in neurodevelopmental disorders in the presence of reduced (or in the absence of) maternal THs because the premature infant's own thyroid production (sufficient for in utero development) is not sufficient to meet its postnatal needs. At full-term birth, TSH levels increase, and T3 and T4 levels then rise through 24 h postbirth, and then begin to decline with adult levels present by 5–7 days postnatal age. The role of the thyroid gland is so important to the in utero and postnatal development of the brain that a case has been made by Howdeshell [21] that the brain is a construct of the thyroid gland.

The hypothalamic–pituitary–thyroid (HPT) axis regulates TH production by a sensitive feedback loop among the hypothalamus of the brain, pituitary, and thyroid gland. TRH from the hypothalamus acts on the pituitary to stimulate release of thyrotropin (TSH), which, in turn, acts on the thyroid gland to initiate TH production resulting in increased circulating T3 and T4. Appropriate levels of circulating T3 and T4 are maintained by negative feedback on the pituitary and the hypothalamus to inhibit production of T3 and T4 in the thyroid gland [22]. Soy isoflavones are estrogenic but they are also goitrogenic (damaging the thyroid and reducing TH output, and thereby stimulating increased growth of the thyroid); iodine deficiency increases soy antithyroid effects, while iodine supplementation is protective of thyroid damage [19].

The *four parathyroid glands* develop in the endoderm, in the paired third and fourth pharyngeal pouches in the neck region; ectodermal and neural crest cells may also be involved. The third pharyngeal pouch forms the inferior parathyroids on each side, and initially descends with the thymus (see later). The fourth pharyngeal pouch forms the superior parathyroids on each side. By Week 6 in utero in humans, the parathyroid diverticulum elongates, at first as a hollow tube, which then fills in from dorsal wall cell proliferation. The fetal parathyroid glands respond to circulating calcium levels, with fetal calcium levels higher than the maternal levels. The parathyroid hormone

(secreted by the chief cells) increases calcium ions, stimulates osteoclasts (bone cells which degrade bone by removing the mineralized matrix), and increases calcium GIT absorption (an opposite effect to calcitonin which decreases calcium GIT absorption); it also controls daily turnover in adult humans of calcium and phosphate.

The *thymus gland* is formed from the endoderm of the third pharyngeal pouch; by Week 6 in utero in humans, the thymus diverticulum elongates, at first hollow, then filled in from ventral cell proliferation, and descends. The thymic primordia are surrounded in vivo by neural crest mesenchyme, triggering epithelial/mesenchymal interactions. The bone marrow lymphocyte precursors in the thymus become thymocytes and subsequently mature into T lymphocytes (T cells). The thymic hormones (thymosins) stimulate the development and differentiation of the T lymphocytes, which are part of the immune system. The thymus and the thyroid glands develop synchronously, in the same region of the embryo at the same time. After maturation to adults, the performance of the cell-mediated component of the immune system declines with age in many mammals and lower vertebrates, accompanied by the involution of the thymus; involution is intrinsic to the thymus gland (an old gland implanted into a young animal will involute, and a young gland implanted into an old animal will survive and thrive until it gets old) [23].

The *pancreas* develops from two pancreatic buds in the endoderm at the intestinal duodenal level; the splanchnic mesoderm forms the dorsal and ventral mesentery in which the dorsal bud forms (the larger of the two and the first to form), and then the ventral bud forms (the smaller and later to form). The development of the pancreas continues with the growth and rotation of the duodenum which brings the dorsal and ventral buds together, where they fuse; the pancreatic duct forms from the ventral bud duct and the distal part of the dorsal bud, this duct has an exocrine function only. The cells of the pancreatic islets are formed by cords of endodermal cells which form ducts from which cells then bud off to form clusters (islets). The exocrine portion of the pancreas takes up 99% of the pancreas by volume, and is comprised of two cell types, the secretory acinar cells and the ductular cells, which produce amylase and α -fetoprotein (and begin to function after birth). The endocrine portion (in the pancreatic islets) takes up 1%–2% of the pancreatic volume, with four different cell types, organized into mixed functional clusters, and functions (by synthesis and release of hormones) starting at 10–15 weeks of age in utero onward in humans. These pancreatic islets (islets of Langerhans) produce endocrine hormones and contain four different endocrine cell types:

1. α -Cells which produce glucagon and mobilize lipid
2. β -Cells which produce insulin and increase glucose uptake; β -cells also stimulate fetal growth in utero, and continue to proliferate into the postnatal period; these cells are most abundant in infancy
3. δ -Cells which produce somatostatin, which inhibits glucagon and insulin secretion
4. F-Cells which produce pancreatic polypeptide

The human timeline for the development of the pancreas is as follows: Week 7 and following in utero: the pancreas forms early, and pancreatic hormone secretion begins and increases, initially with a small amount of maternal insulin; Week 10: α -glucagon is produced first, then δ -somatostatin is produced; insulin-producing β -cells differentiate and fetal insulin secretion begins; Week 15: glucagon is first detectable in fetal plasma.

The *paired adult adrenal glands* are richly vascularized with arterioles passing through the cortex, and with capillaries from the cortex to the medulla in a portal-like circulatory pattern.

The fetal adrenal cortex produces a steroid precursor, dehydroepiandrosterone (DEA) which is converted by the placenta into estrogen. It also produces hormones which influence lung maturation. The adrenal cortex derives from the coelomic epithelium and its hormones are lipid-soluble steroids: cortisol, aldosterone, and DEA. The adrenal cortex differentiates to form cortical zones in the late fetal period. The zona glomerulosa is regulated by the renin–angiotensin–aldosterone system which in turn is controlled by the juxtaglomerular apparatus of the kidney; the zona fasciculata is regulated by the hypothalamic–pituitary axis by the release of CRH and ACTH. The fetal adrenal cortex is later replaced by the adult cortex. In the sixth week in utero, the human fetal cortex is formed from the mesothelium adjacent to the dorsal mesentery; the adult adrenal cortex forms when mesothelial mesenchyme encloses the fetal cortex. At birth, the human adrenal cortex is composed of two zones, the zona glomerulosa and the zona fasciculata; by the third year of life, the third zone, the zona reticularis, is present.

The hypothalamic–pituitary–adrenal (HPA) axis responds to stressful situations as follows: the brain receives information on the stress situation from sensory inputs, the hypothalamus releases CRH, which goes to the pituitary gland which then releases ACTH into the blood, which goes to the adrenal glands to trigger production and/or release of cortisol, which triggers responses in appropriate organs/limbs (e.g., “the flight-or-flight response”) to respond to the stressor(s).

The fetal adrenal medulla is formed from neural crest cells from adjacent sympathetic ganglia, when the neural crest cells migrate adjacent to the coelomic cavity; initially the fetal medulla is uncapsulated and not surrounded by fetal cortex; the medullary cells at this time have a neuron-like morphology [24]. There are two cell types in the adrenal medulla: one cell type secretes the amino acid derivative epinephrine (adrenaline, 80%) and the other cell type secretes norepinephrine (noradrenaline, 20%) in adult vertebrates. The medulla is actually considered a neuroendocrine transducer rather than an endocrine gland, since it secretes its product in response to neural input via acetylcholine released at synapses by preganglionic sympathetic neurons [25].

The *gonads* (both ovaries and testes) develop from the mesoderm forming the mesothelium and underlying mesenchyme and the primordial germ cells; the gonadal ridge is formed by the thickening of the mesothelium in the medial

mesonephros; the primordial germ cells are formed in the yolk sac, move to the mesentery of the gut and then to the genital ridge of the developing kidney [26]. The development of the testes or the ovaries is dependent on the presence (testes) or absence (ovaries) of the testis-determining factor (TDF) on the Y-chromosome (found only in males).

By 7–8 weeks in utero, the testis, which develops from mesothelium and mesonephros, initially forms a mass covered by surface epithelium. In the central mass, a series of cords appears, and the periphery of the mass converts into the tunica albuginea. The cords grow together toward the future hilum and form a network, termed the rete testis. The seminiferous tubules, site of future sperm production, form from these (seminiferous) cords. The interstitial cells of Leydig (in the developing testis outside of these seminiferous tubules) begin to secrete testosterone and androstenedione. By 8–12 weeks in utero in human males, human chorionic gonadotrophin (hCG), a glycoprotein produced in pregnancy (initially by the developing embryo and later by the syncytiotrophoblast, which is part of the placenta), stimulates testicular Leydig cell testosterone production; the testicular Sertoli cells (a kind of sustentacular or “nurse” cell) produce anti-Müllerian hormone (AMH, to suppress female hormone production) from this point, in utero, to puberty in the male. Later, postnatally just prior to puberty, the testes descend, pulled by the gubernaculum cord (or gubernaculum), into the scrotal sacs, presumably to prevent overheating of the testes and destruction of the temperature-sensitive mature spermatozoa after puberty. Exposures to exogenous androgens and environmental anti-androgens in utero have been shown to affect reproductive development and sex-specific play behavior in rats [27].

In the future ovary, the primordial germ cells also form in the yolk sac and multiply and migrate; once they reach the gonadal ridge, they are termed oogonia (the diploid stem cells of the ovary), by about the seventh week in utero in humans. The oogonia associate with other somatic cells from the peritoneum and the mesonephros (and/or possibly the mesothelium). These cells, likely the pre-granulosa cells (although the embryological origin of the granulosa cells is “controversial”; see earlier) form a layer of connective tissue cells around each oogonium to become the ovarian follicle [28]. The ovary is formed initially as a mass of cells mainly from the genital ridge and the mesonephros [29]; the mass then differentiates into the central medulla covered by the germinal epithelium; the larger immature ova are found between the germinal epithelial cells. The surface germinal epithelium forms the permanent epithelial covering of the ovary, loses its connection with the central mass, and the tunica albuginea forms between them. The ovaries produce 17β -estradiol, estradiol, estrone, estrogen, and progesterone. A short gubernaculum cord also forms in the female which causes a shift in the position of the ovaries to the base of the uterus.

The *placenta* begins when the embryo-derived trophoblast cells from the recently implanted blastocyst (Day 6 of gestation in humans) [30] invade the uterine lining, and cells of the uterine endometrium prepare for this invasion [31]. The placenta is composed of maternal (decidua) and fetal

(trophoblastic cells and extraembryonic mesoderm) components. The human placenta (and all other mammalian placentas) makes a number of hormones:

1. hCG which supports the ovarian corpus luteum (or corpora lutea in the case of multiple conceptuses), and maintains the pregnant status rather than the menstrual status (which would cause the uterine lining to be shed)
2. Human chorionic somatotropin (hCS, or human placental lactogen, hPL) which stimulates maternal mammary gland development
3. Human chorionic thyrotropin (hCT)
4. Human chorionic ACTH (hCACTH)
5. Progesterone and estrogens which support the maternal endometrium
6. Relaxin, a protein hormone from the insulin superfamily

In males, relaxin enhances sperm motility. In nonpregnant and pregnant females, relaxin is produced by the corpora lutea of the ovary, formed after eggs are ovulated; in nonpregnant females, it peaks at 14 days after ovulation and then declines, triggering sloughing of the uterine lining and menstruation. It is also produced by the maternal placental decidua during pregnancy. Its function during pregnancy is to allow expansion of the uterus (the first peak in hormone level is at 14 weeks, the end of the first trimester), and to soften the pubic symphysis, widening the gap between the pubic bones in the lower ventral abdomen to facilitate labor and delivery. The placenta also synthesizes and secretes the maternal and fetal precursors of the protein hormones: hCG, hCS or hPL, hCT, and hCACTH. The hormone hCG is produced up to 20 weeks of pregnancy and stimulates the fetal adrenal cortex growth, differentiation, and maintenance; hCS rises throughout pregnancy and also stimulates maternal metabolic processes and breast growth. The placental steroid hormones include progesterone to maintain pregnancy, and estrogens to support the fetal adrenal gland and the placenta itself.

There are *other endocrine hormone sources*. They include

1. The *endocrine heart* which produces
 - a. Atrial natriuretic peptide (ANP) to increase renal filtration rate and decrease renal sodium ion reabsorption
 - b. Endothelins (ET-1, ET-2, and ET-3) to control vasoconstriction, and to increase nitric oxide (NO) which in turn causes vasodilation
2. The *endocrine kidney* which produces
 - a. Renin to increase the angiotensin–aldosterone system
 - b. Prostaglandins to decrease sodium ion reabsorption
 - c. Erythropoietin to increase erythrocyte (RBC) production
 - d. $1,25(\text{OH})_2$ vitamin D to control calcium homeostasis
 - e. Prekallikreins to increase kinin production

The kinin–kallikrein *system* is a poorly understood system of blood proteins, which plays a role in inflammation, blood pressure control, coagulation, and pain; its mediators, kinin and kallikrein, are vasodilators which act on many cell types [32,33]. The urine has a high concentration of kinins which leads to hypotension (low blood pressure).

3. The *endocrine GIT* which provides enteric control of digestive function, including production of five hormones:
 - a. Gastrin secreted by the G-cells of the stomach which controls gastric acid secretion
 - b. Ghrelin, made in the stomach wall, which triggers feelings of hunger (in dieters who lose weight, ghrelin levels increase to stimulate eating); ghrelin and leptin (see later) act oppositely to keep food intake and weight in balance
 - c. Cholecystokinin, a small intestinal hormone which stimulates secretion of pancreatic enzymes and bile
 - d. Hormone PYY, secreted by the small intestine after eating, which acts as an appetite suppressant to counter the appetite stimulant, ghrelin
 - e. Secretin, formed by the epithelial cells of the small intestine, which stimulates secretion of bicarbonate-rich fluids into the small intestine from the pancreas and liver
4. The *adipose tissue* which produces three hormones
 - a. Leptin, a polypeptide hormone produced in adipose cells and many other tissues, which suppresses appetite as its level increases; when body fat decreases, leptin levels fall and appetite increases
 - b. Adiponectin which regulates energy homeostasis and glucose and lipid metabolism, as well as acting as an anti-inflammatory on the cellular vascular wall
 - c. Resistin (for observed resistance to insulin in mice injected with resistin), a polypeptide of 108 amino acids (in humans) and the related resistin-like protein β (resistin-like molecule β), hormones secreted by adipocytes

Evidence from early studies suggested a correlation between blood glucose levels and blood resistin concentration in mice, thereby providing the link between obesity and diabetes mellitus, type 2 [34]. However, more recent studies do not show increases in blood resistin levels in obese humans with diabetes [35,36]. Research continues on the role(s) and importance of resistin in the body (see Table 12.2).

EVOLUTION OF THE VERTEBRATE ENDOCRINE SYSTEM

Campbell et al. [37] stated in the introduction to their paper that “One of the great challenges in biology is to understand how particular complex morphological and physiological characters originated in specific evolutionary lineages.”

They proposed that the origin of the highly conserved vertebrate hypothalamic–pituitary–peripheral gland (HPPG) endocrine system arose by emergence of complex features by genetic changes/modifications in which the preexisting ligand–receptor systems were “bridged” (modified) to form new networks. These innovations could include changes in gene expression, and therefore structural innovations to proteins that altered linkages in metabolic and signaling pathways. They reasoned that genes from vertebrate systems with homologs in invertebrates were more likely present in ancestral chordates; vertebrate features not present in invertebrates could be from key innovations occurring after the divergence of the vertebrates from the invertebrates. For example, vertebrate pituitary hormones are absent in invertebrates. (They looked at ascidians, genus *Ciona*.) This could have occurred through gene innovations in early vertebrates (as they proposed), or by gene loss from the invertebrate genomes. Interestingly, Campbell et al. [37] did note that recent analyses of expressed sequence tags (ESTs) from cnidarians revealed “a relatively high proportion and a high frequency of gene loss” in these invertebrates. However, Campbell et al. [37] still proposed that the absence of the pituitary hormones and receptors “from all of the available invertebrate sequences supports the interpretation that these genes were innovations of the vertebrate lineage.” They therefore proposed that the vertebrate HPPG endocrine system arose by regulatory and structural modifications to protein ligands, receptors, and enzymes present in the last common chordate ancestor (amphioxus), perhaps by “read-through mutation or alternative exon splicing” in the genes for these proteins.

The evolutionary steps resulting in the components of the vertebrate endocrine system have been worked out (to a greater or lesser degree) for all of the major vertebrate hormones now known. The current views of their evolution follow.

The acquisition of a hypothalamic–pituitary axis is considered the first step and perhaps the most critical event in vertebrate evolution leading to neuroendocrine control of growth, reproduction, osmoregulation, stress, and metabolism [1]. Cephalochordates (e.g., amphioxus), urochordates, and vertebrates are presumed to have evolved from a common ancestor over 520 million years ago [38]. In these groups, there are three major axes, operating by negative feedback inhibition: the HPG axis, the HPT axis, and the HPA axis, with pituitary glycoproteins used in both the HPG and HPT axes. These axes are clearly separated and distinct in more advanced vertebrates; they are less specified in evolutionarily older taxa [4].

The HPG axis: The cephalochordate amphioxus (an ancient invertebrate chordate) is considered the most primitive chordate; it does not have a structurally identifiable endocrine system comparable to the HPG axis, but recent genomic analyses indicate the presence of the thyrostimulin gene. The thyrostimulin gene product is a glycoprotein hormone (GPH, with α - and β -subunits), present in various organs in subsequent vertebrates, including humans [39].

Thyrostimulin homologs have been recently discovered in invertebrates, suggesting that this most ancestral heterodimeric GPH existed even before the divergence of the vertebrates and invertebrates [40]. It is viewed as an ancestral precursor of the GPHs found in chordates. In genomic analyses, genes for sex steroid hormones were also found in amphioxus, and the conversion pathways of the sex steroids to estrogen, androgen, and other major sex steroids were also identified in the amphioxus gonads, evidence for a primitive endocrine reproductive system in this ancient prevertebrate chordate [41]. Amphioxus also has a nuclear receptor gene complement which mediates the cellular responses to certain key hormones, a complement close to that of other subsequent chordates [42].

The hagfish, which lacks both jaws and vertebrae, is considered the first, most primitive vertebrate known, living or extinct. However, the hagfish does synthesize a functional GPH, with two subunits (α and β) synthesized and colocalized in the same cells of the hagfish adenohypophysis (i.e., the anterior lobe of the modern pituitary). The cellular and transcriptional activities of these subunits are correlated with the developmental stages of the gonad. Uchida et al. [43] hypothesized that the identification of this single functional GPH in the hagfish provides critical evidence for the likelihood of a pituitary–gonadal system in the earliest divergent vertebrates (evolving from an ancestral prevertebrate chordate; see earlier). In addition, recent evidence indicates that the hagfish also produces gonadotropin (GTH), ACTH, and GH [44]. However, a structure designated the pituitary gland has not (yet) been found in the Protochordates [43]. The proopiomelanocortin (*POMC*) gene, coding the synthesis of a number of hormones (see later), was most likely derived from an ancestral opioid-coding gene following the first major chordate genome duplication event in hagfish. During the radiation of the jawless hagfish, the *POMC* organization plan emerged with multiple melanocortin sequences (α -MSH/ACTH and β -MSH) and a C-terminally extended opioid sequence (β -endorphin [β -END]). Following the second major genome duplication in early vertebrates, the γ -MSH sequence was acquired.

Among the jawed vertebrates (beginning with the lamprey, see later), three distinct trends in the evolution of the *POMC* gene appeared. They were

1. Gain of the δ -MSH sequence in cartilaginous fish
2. Loss of the γ -MSH sequence in ray-finned fish
3. Retention of the post second major genomic duplication of the *POMC* organization plan in both lobe-finned fish and tetrapods

POMC is synthesized in the pituitary gland, and in neurons of the hypothalamus of the brain, where an array of post-translational processing mechanisms, including endoproteolytic cleavage and N-acetylation, produces distinct sets of end products in these tissues. A striking feature of the melanocortin end products is the rigorous conservation of the primary

sequence of α -MSH and the first 25 amino acids of ACTH from hagfish to higher vertebrates, including mammals [45].

The next evolutionary step occurred in the lamprey, a basal vertebrate, with two GTH-releasing hormones (GnRHs) with functional roles acting via the HPG axis, controlling reproductive function [1]. Sower et al. [1] identified a novel glycoprotein receptor, designated lamprey GnRH-II (GnRH-like receptor). They hypothesized that the lamprey GnRH-II is an ancestral GnRH to the later vertebrate GnRHs. A second glycoprotein receptor, IGpH-RII, was also identified in the lamprey, suggesting the existence of a “primitive, overlapping yet functional” HPG and HPT endocrine system in the lamprey, involving one or two pituitary GPHs and two GPH receptors [1,46,47]. The hormones identified in the sea lamprey are therefore characterized as adenohypophysial hormones.

The recent use of genomics indicates that the peptide sequences of GnRH and its receptor are also present in invertebrates from amoebas to tunicates, with structural conservation of a GnRH-like protein of 10–12 amino acids. This, in turn, suggests homology between the 15 known invertebrate GnRH-like peptides and the 15 known vertebrate GnRHs. Roch et al. [48] suggest a superfamily that includes GnRH, insect adipokinetic hormone (AKH), and corazonin (*Drosophila* G protein-coupled) receptors closely associated with the vasopressin/oxytocin superfamily of receptors. The functional role of the GnRHs in invertebrates as well as in vertebrates suggests (to Roch et al. [48]) that a functioning pituitary was likely the result of genomic duplication in early vertebrates.

The glycoprotein proteins (GTHs) are core members of the ancient GPH family [49]. They include TSH, FSH, LH, and CG (chorionic GTH), which are found in the pituitary in thyrotrope and gonadotrope cells in vertebrates, and in the placenta in mammals. They are derived from multiple sources but they have highly conserved functionality across classes, genera, and species, and stimulate gonadal activity and steroidal release in these groups [4]. For example, chorionic GTHs are produced by the primate fetus, placenta, and pituitary gland [50] but hCG is also an effective GTH in amphibians [51], fish [52], and reptiles [53]. The *TSH β* gene, expressed in gonadotropes in conjunction with the *TSH α* gene, is subject to transcriptional repression by TH; the *FSH β* gene, also expressed in gonadotropes, is controlled by activin and inhibin (regulated by GnRH); and the *LH β* gene is expressed also in gonadotropes, dependent on GnRH, but unaffected by activin or inhibin. The *CG β* gene is viewed as most recently evolved from the *LH β* gene and is expressed in the placenta, rather than in the pituitary, in placental mammals.

The adenohypophysial hormones are believed to have evolved from several ancestral genes by duplication followed by divergence. In the sea lamprey, ACTH, melanotropin (MSH), and β -END are encoded in two distinct genes, proopiomelanocortin (POC) and proopiomelanotrophin (POM), expressed in the RPD and PA, respectively, of the pituitary gland, the same organization followed for all subsequent vertebrates (except for jawed fishes). In jawed fishes (gnathostomes), ACTH and

MSH are encoded in a single gene. POMC also evolved in invertebrates by duplication of the MSH domains. The ancestral *POMC* gene in the early vertebrates, lobe-finned fishes and tetrapods, resulted from internal duplication and deletion of the MSH domains, from the POMC of the lamprey and gnathostome fish. The sea lamprey GH is found in the proximal pars distalis of the ancient pituitary gland, and is stimulated by exposure to IGF in the liver, as in other vertebrates. GH is the only member of the GH family in the lamprey; it is assumed to be the ancestral hormone, first in the molecular evolution of the GH family in vertebrates. Both PRL and somatolactin (SL) also presumably evolved from gene duplication. After the agnathans, gonadotrophic hormone (GTH) β in the lamprey was likely duplicated to form GTH and TSH in the lamprey and subsequent vertebrates [54].

In vertebrates, activation of the hormone GnRH and its GnRH receptor in the pituitary gland is necessary to activate the reproductive cascade including production and release of GTHs, LH, and FSH in mammals, into the blood supply for transport to the gonads where they regulate steroidogenesis and gametogenesis [55]. GnRH-binding sites have been detected in the mammalian brain, testicular Leydig cells, the placenta, ovarian luteal and granulosa cells, adrenal cortex, immune tissues including the thymus (see later), spleen, blood lymphocytes, and other tissues. The HPG function of GnRH is highly conserved, but possible alternative roles for GnRH are not well understood, example, the presence of GnRH-II in the midbrain of most vertebrates and its association with sensory and motor functions implies that it is (or may have been) important in neural regulation. It is known that interruption of GnRH function produces sterility in all vertebrate classes and that the same pattern of GnRH expression is present in brains across all vertebrate classes [4]. It is also known that GnRH is a highly conserved peptide that is expressed early in brain development in vertebrates. In zebra fish, GnRH was detected in the embryo within 2 h after fertilization. Since loss/knock-down of either GnRH2 or GnRH3 affects early regionalization of the brain and eye development, Wu et al. [56] suggested that GnRH plays a major role in early brain regionalization and eye development (at least in zebra fish).

However, less is known about GnRH receptors prior to the evolution of the vertebrates. There is strong homology between functional GnRH peptides and receptors in vertebrates and tunicates (Craniata and Urochordata) [4]. In fact, discovery of a receptor for octopus GnRH with sequence and functional similarity to vertebrate GnRH receptor implies a common GnRH ancestor between chordates and proto-stomes [4]. Genome sequencing was recently completed for amphioxus (a very ancient invertebrate chordate; see earlier). Tello and Sherwood [57] cloned and characterized four GnRHR cDNAs from the amphioxus genome. They reported that a mammalian GnRH-I and a chicken GnRH-II incubated with COS7 cells expressing the amphioxus GnRHRs resulted in potent intracellular inositol phosphate turnover in two of the receptors, one of them preferring GnRH-I over GnRH-II (such preference was previously only observed in type 1 mammalian GnRHRs). Strikingly, the four amphioxus

receptors grouped into two paralogous pairs by phylogenetic analysis, one pair grouping with the vertebrate GnRH receptors and the other pair grouping with the invertebrate/octopus GnRHR-like sequences and the related insect ADK hormone. Octopus GnRH-like receptor and insect ADK hormone also induced potent inositol phosphate turnover in one of the other two amphioxus receptors. Therefore, the authors concluded that there is functional conservation of two distinct types of GnRH receptors at the origin of the chordate lineage (in the amphioxus). The authors propose that the amphioxus “vertebrate-like” receptor pair began the evolution to the vertebrate GnRHRs, and that the other “octopus/insect-like” receptor pair (also related to the Mollusk GnRHR-like receptor) was apparently lost in the evolution of the vertebrate lineage.

All vertebrates studied before 2008 have 1–3 forms of GnRH in specific different neurons in the brain. At least one type of GnRH receptor is also present in each vertebrate. Humans have two types of GnRHs: GnRH1 and GnRH2, but only one functional GnRH receptor. Zebra fish also have the same two types of GnRH receptors (1 and 2), but they have four different GnRH receptors in their genome. Tello et al. [58] cloned the four GnRH receptor cDNAs from a single species of zebra fish. The four receptors are G-proteins (heterodimeric guanine nucleotide [GDP, GTP]-binding proteins, the most common method of signaling in cells), with 45%–71% amino acid identities in common. High sequence similarity was also observed with human GnRHR, green monkey type II GnRHR, and two goldfish GnRHRs. The zebra fish receptors are expressed in the brain, eyes, and gonads. In the inositol phosphate assay, all four receptors were functional, based on the response to physiological doses of native GnRH peptides, with two of the receptors exhibiting selectivity between GnRH2 and GnRH3. The four receptors map to four different chromosomes, and are segregated into distinct phylogenetic groups, with separate gene lineages conserved throughout vertebrate evolution. The authors suggest that the retention of four functional GnRH receptors in the zebra fish (versus only one GnRH receptor in humans) may be due to/depend on “subfunctionalization” (one or more forms acquire separate but complementary functions over evolutionary time) and/or “neofunctionalization” (one or more forms acquire new distinct functions over evolutionary time) in the zebra fish. The differences in structure, location, and response to GnRH forms “strongly suggest” to Tello et al. [58] that the four zebra fish receptors have therefore acquired novel functions over evolutionary time, in addition to the conventional activation of the pituitary gland in the reproductive axis.

Kisspeptin (a product of the *Kiss1* gene) and its receptor (GPR4 or Kiss11r) have recently been recognized as key players in the brain regulation of reproduction in vertebrates [59]. Neurons expressing Kiss1/kisspeptin are localized in discrete nuclei in the hypothalamus and in other brain regions; their distribution, regulation, and function vary widely across species. Kisspeptin neurons differ by sex with respect to cell numbers and transcriptional activities in certain brain nuclei, and some of them express other cotransmitters as well.

Kisspeptin neurons also express the estrogen and androgen receptors (ARs), so these cells are responsive to gonadal steroids in both sexes. Kisspeptin signaling in the brain is likely involved in the negative feedback of sex steroids on GTH secretion, generating the preovulatory GnRH/LH surge, triggering and regulating the process of sexual maturation and puberty, controlling seasonal reproduction, and inhibiting reproductive activity in the female during lactation. Mutation in humans and genetically targeted deletions in mice of either *Kiss1* or *Kiss1lr* result in hypogonadotropic hypogonadism (profoundly decreased bilateral gonadal size from greatly reduced GTH production/secretion). Kisspeptin signaling may also extend outside of reproductive neuroendocrinology, including regulation of metastasis in certain cancers, vascular dynamics, placental physiology, and even higher-order brain function [59].

By 2010, the *Kiss1* gene product kisspeptide was considered an essential regulator of the HPG axis in most vertebrate species. Recent studies have reported the existence of paralogous kisspeptin and kisspeptin receptor genes in fish as well as in amphioxus. Fish are therefore being used as an animal model to study the general principles underlying the evolution of the kisspeptin and the kisspeptin receptor system in vertebrates. Unlike placental and marsupial mammals, many teleost species have two paralogous genes of kisspeptin: *Kiss1* and *Kiss2*. Medaka (*Oryzias latipes*), with *Kiss1* and *Kiss2* genes expressed in distinctive hypothalamic neuron populations, is considered a good model system of central regulation of reproduction. In the medaka, the *Kiss1* system (but not the *Kiss2* system) exhibits expression dynamics indicative of its direct involvement in HPG axis regulation by its actions on GnRH1 neurons [60]. However, in some fish species, the *Kiss1* gene is missing and only *Kiss2* is expressed, so the *Kiss2* peptide is likely the regulator of reproduction in these fish. The ancestral vertebrates probably had the two paralogous *Kiss* genes involved in HPG axis regulation. In the subsequent species which retained both paralogs during evolution, either *Kiss1* or *Kiss2* retained its role in HPG regulation while the other *Kiss* protein assumed nonreproductive functions (neofunctionalization, see earlier). Alternatively, both paralogs might have assumed complementary functions in HPG axis regulation (subfunctionalization). After the divergence of the teleost and tetrapod lineages, either one of the two paralogs (or both in birds) has been lost (degradation) or has become a pseudogene (nonfunctionalization), but the remaining paralog (if present) has retained its original function of HPG regulation. The identification of multiple forms of kisspeptin receptor systems and the “rather promiscuous” ligand–receptor relationship have been proposed as the bases for the functional robustness of kisspeptin and kisspeptin receptor systems in HPG regulation, even when one or both paralogous genes are lost or become functionally divergent during evolution [61].

The entire SN sequence, expressed in the pituitary gland, exhibits high sequence similarity among mammalian species, such as human, pig, hamster, and rat, and is somewhat conserved in nonmammalian species such as amphibians (with

two regions identical to those of mammalian SN), and sharks. Teleost (bony fish) SN is less similar to the mammalian SN, but has the same number of amino acids. In the zebra fish, there are two genes, likely derived from gene duplication: the *Sg11a* gene is located on chromosome 15, and the *Sg11b* gene is located on chromosome 2; the gene products of *Sg11a* and *Sg11b* have conservation of 11 of 33 (33%) amino acids in the middle region, relative to the other vertebrates tested to date including humans. Sg11 precursor is stored with other secretory products (essentially hormones) in pituitary cells of all animals evaluated. The granins, including Sg11, localize with GH and PRL in the rat mammotrophs in the pituitary gland, whereas Sg11 is differentially colocalized in secretory granules in the bovine (cow) pituitary gland with TSH and LH [18].

The pineal gland serves as a photoreceptive neuroendocrine organ in many lower vertebrates, with both morphological and functional similarities between the pineal and retinal photoreceptor cells (e.g., the presence of diminutive stalked eyelike structures and a pineal foramen [opening] in the skull of ancient vertebrates, likely used for photoreception in the pineal complex in these ancient lower vertebrates). These two organs, the pineal gland and the eye, are viewed as having a close evolutionary relationship, with divergence in the pineal gland from the “median third eye” early in vertebrate evolution with the progressive loss of photoreception in higher vertebrates (above amphibians) and its development into a gland which synthesizes melatonin. Melatonin from the pineal gland in higher vertebrates plays a major role in sexual development, hibernation, metabolism, seasonal breeding, and circadian rhythms [62]. The pineal gland is also viewed by Dr. R. Strassman, a psychiatrist and clinical pharmacologist, as the site of synthesis of *N,N*-dimethyltryptamine (DMT), a powerful psychedelic (“the Spirit Molecule”). The required methyltransferases to make DMT are found in the pineal gland (although critics point out that the specificity of the enzymes allows only the conversion of serotonin to melatonin). In 2001, Strassman conjectured that when someone is near death or in a dream state, the pineal gland releases large amounts of DMT, causing the images reported by survivors of near death experiences or by volunteers ingesting large amounts of DMT [63].

Phylogenetically, thyroid cells are viewed as derived from primitive iodide-concentrating gastroenteric cells. With the movement of early organisms from iodine-rich oceans to iodine-deficient fresh water areas and on land, these organisms likely required better systems to concentrate and retain iodine. Venturi et al. [64] suggested that iodide functioned as an ancestral antioxidant in iodide-concentrating cells from primitive algae to more recent vertebrates. Recently, Kupper et al. [65] experimentally confirmed that iodides did act as ancestral antioxidants, even as early as 700 million years ago. Iodine was (and is) present, while exhibiting no hormonal activity, in the fibrous exoskeletal scleroproteins of the earliest invertebrates, Porifera and Anthozoa. The active vertebrate hormone, triiodothyronine (T3), evolved roles in metamorphosis, example, from relatively modest changes for the transition from

water-based life to land-based life in urodeles (e.g., salamanders) involving resorption of the tail fin, destruction of the external gills, and changes in skin structure, to major changes in anurans (e.g., frogs and toads) involving loss of the tail, growth of hind limbs, loss of tadpole teeth, loss of internal gills, major diverse biochemical changes including switching from excretion of ammonia in tadpoles (and in freshwater fish) to excretion of urea in adult frogs (and in other terrestrial vertebrates [3], and in thermogenesis, to regulate heat production in organisms exposed to temperature fluctuations, enabling better adaptation of organisms to a fresh water or a terrestrial environment, with fresh water (low iodine), atmosphere, gravity, temperature fluctuations, and varied diets.

In tetrapods, the parathyroid glands play a major role in regulating extracellular calcium homeostasis, which, in turn, is important for many physiological processes such as muscle contraction, blood coagulation, and synaptic activity. These glands detect changes in calcium levels in the blood by the calcium-sensing receptor (CasR) which modulates the secretion of parathyroid hormone, which, in turn, acts to release calcium from internal stores such as bone, and modulates renal calcium ion transport. In mice (so far), the transcription factor encoded by *Gcm2* (a mouse ortholog of the *Drosophila* glial cells missing gene) is a key regulator of parathyroid gland development; if this gene is mutated prior to/during early development, the parathyroid glands do not form. In contrast, fish are believed not to have parathyroid glands or parathyroid hormone; unlike tetrapods, the majority of calcium uptake in fish is from external sources (the ocean) via the gills, although both fish gills and tetrapod parathyroid glands express *Gcm2* (see later). These differences appear to coincide with the evolution of tetrapods and the shift from an aquatic to a terrestrial environment, in which new controls were necessary for regulating calcium homeostasis; so the evolution of the parathyroid gland and the production of parathyroid hormone were keys to internally regulate serum calcium levels on land. A phylogenetic analysis was performed by Okabe and Graham [66]. Their results indicated that the parathyroid glands of tetrapods and the gills of fish share a common evolutionary origin, with both expressing *Gcm2* and requiring this gene for their formation, and both expressing parathyroid hormone and CasR. The authors suggest that the parathyroid gland developed as the result of the transformation of the gills of fish in water into the parathyroid glands of tetrapods on land. *Gcm2* is found throughout the jawed fishes (gnathostomes), including Australian lungfish, trout, zebra fish, and dogfish, and in birds and mammals. The *Gcm2* gene is expressed in the pharyngeal gill pouches and their derivatives, the internal gill buds, in chondrichthyan (cartilaginous fish), and teleost (bony fish) species, and was present before the emergence of bony fishes; it persists as an ancient conserved feature of all jawed vertebrates (including mammals). It is specifically required in fish for the formation of internal gill buds from the pharyngeal pouches and in tetrapods, including mammals, for the development, differentiation, and survival of the parathyroid gland, strongly suggesting that these structures are closely related. Also in

support of this view is the finding that the parathyroid hormone–encoding gene is present both in tetrapod parathyroid precursor tissues and in teleost fish gill tissues [66].

A comparison of the embryology and evolution of the thymus in the Chordata, starting with the Protochordates (Acrania) through all classes of Vertebrata including humans by Slipka et al. [67], indicates that it is likely that the whole branchial region (with its gills and clefts) initially arose to provide nutrition. In the early stages of evolution in Chordata, it apparently became inadequate to efficiently filter food passively through the gills and to respire wholly through the skin. Tongue bars occurred in the gill slits of amphioxus (and now in only one other organism, the worm-like *Balanoglossus* [68]), likely to increase surface area. The highly vascularized gills became able to regulate the internal environment with regard to gas (O_2/CO_2) exchange and control of ions. The clefts in the protochordate branchial region became organs of excretion, eliminating extraneous materials and waste. In the higher vertebrates, the tongue bars are absent, but in the development of the thymus in higher vertebrates, there is a structure which arises from the dorsal half of the gill slits, which may be the homolog of the amphioxus tongue bars. From this initial primitive system, a more advanced system arose in more complicated organisms: lymph sinuses in current cyclostomes and the first thymuses in sharks. This structure developed into the ductless thymus gland, which has undergone extensive changes during evolution, including transition from an ancient active function to an apparently passive function in existing Craniota [68]. This system of immune reactivity was tightly associated with the advancement of the lymphoid system and the thymus apparently developed simultaneously with the thyroid [69].

Comparative morphological and immunohistochemical studies in the 1980s enabled a detailed reconstruction of pancreatic evolution. Evolutionarily, in insects (considered the most evolved of the invertebrates), only the brain cells produce gastrointestinal hormones. Protochordates, closely related to the subsequent vertebrates, have both exocrine and endocrine cell types in their digestive tract mucosa; the cells are dispersed and do not form functional clusters. The newer (more advanced) vertebrates, including amphibians, reptiles, birds, and mammals, have the same gross anatomy of the pancreas, while different fish species vary in both complexity and quantity of pancreatic structures, likely reflecting different steps in early vertebrate pancreatic evolution. The Atlantic hagfish, the most primitive vertebrate known, has a primitive pancreatic organ with lobular cell nests closely associated with the bile duct. In the bile duct are numerous cells producing insulin (absent from the gut in higher vertebrates), as well as a few cells producing somatostatin; there is no exocrine function and no acinar cells in hagfish [70]. A primitive exocrine pancreas is present in cartilaginous fish, with a pancreatic duct directly ending in the gut lumen, connected to a glandular structure made of exocrine cells, associated with cell islets in which there are three different hormone-producing cell types, making insulin, glucagon, and somatostatin [71].

The pancreatic gland, providing both exocrine and endocrine functions in separate cells and layers, is specific to the vertebrates. The functional organization of the pancreas in higher vertebrates is first represented by the cartilaginous fish (sharks and rays), in which the endocrine islets contain the four hormone-producing cell types, including PP cells which produce PP (pancreatic peptide); the cells lining the pancreatic lumen fold to form the ductular pancreas. In teleosts (bony fishes), the four types of endocrine cells are organized into anatomically discrete islets. With the exception of insulin, the pancreatic polypeptides are still located in cells dispersed throughout the GIT in bony fish. The evolution of the epsilon cells in the pancreas, which produce ghrelin, is not yet determined [70].

The cortex of the adrenal gland is the endocrine component of the gland, synthesizing and secreting the steroidogenic compounds, corticosterone, cortisol, and aldosterone, beginning with the jawed fishes and continuing, with very similar biosynthetic pathways, through to the eutherian mammals [72]. The adrenal gland has a close evolutionary relationship with the mesonephros, a primitive kidney structure, and the metanephros, a more advanced kidney structure. Close examination of amphibian urodele and anuran adrenal glands by Milano and Accordi [73] indicated that urodeles (the earlier/older family, e.g., salamanders) exhibited large variability in adrenal glandular structure without a clear taxonomic pattern, although increased compactness of the gland and intermingling of steroidogenic and chromaffin cells are found in some more advanced neuroleles. In more recent anurans (e.g., frogs and toads), the glandular pattern is divided into two subtypes: one more medial and diffuse (in more primitive families) and the other more lateral and aggregated (in more advanced families). The adrenal gland therefore increased its compactness and aggregation of chromaffin and steroidogenic tissues as the class evolved from primitive to advanced families, in both urodeles and anurans. This initial similarity in gland morphogenesis across all amphibians continues until the end of metamorphosis (e.g., the structural and functional changes from aquatic-based life to terrestrial-based life). After metamorphosis is complete, changes in glandular structure and location continue only in anurans until the gland reaches its final form and position [73]. Mammals are unique among vertebrates in that the adult adrenal gland is organized into discrete layers [25].

The vertebrate gonads (both ovaries and testes) produce not just eggs and sperm, but also synthesize many peptides that were initially classified as neuropeptides, since they were found initially to be produced in neural cells. In fact, many neuronally produced peptides with important endocrine, behavioral, sensory, and autonomic roles in the vertebrate brain are also expressed by and may act directly within the gonads, example, neuropeptide Y, bradykinin, corticotropin-releasing factor (CRF), GnRH, GTH-inhibitory hormone (GnIH), and Kisspeptin; the gonads also have the appropriate hormone receptors. There is also evidence that the gonads can and do respond to environmental and physiological cues and regulate their own steroid production, estrogens and

androgens [74]. Evidence for the beginnings of a reproductive endocrine system in hagfishes has been reported [41].

In mammals, inhibin and activin are produced by the gonads as well as the fetoplacental unit and the pituitary gland [75]; they are closely related disulfide-linked dimers belonging to the transforming growth factor- β superfamily. (See the previous section on the development and functions of the pituitary gland for more information on activin and inhibin.) To determine whether activin and inhibin are present and have function(s) in (nonplacental) lower vertebrates, Poon et al. [76] used a zebra fish model in which an inhibin-specific α -subunit ("inha") is expressed in the gonads, with no transcripts found in the pituitary or brain. In the ovary, inha is expressed in the somatic cells of the follicle, not in the ova or in ovulated unfertilized eggs. Inha levels rise during folliculogenesis and drop during oocyte spontaneous maturation and during germinal vesicle breakdown. Goldfish pituitary extract and forskolin both stimulate inha expression. Recombinant zebra fish FSH, but not LH, also significantly increases inha expression. The stimulation of inha expression by FSH and the inhibition of FSH by inha suggest the existence of a negative feedback loop between the pituitary (FSH) and the ovary (inhibin) in the zebra fish [76]. The striking concordance of embryological development and signaling pathways between zebra fish and mammals suggests the possibility that such a feedback loop between the pituitary and the ovary may also exist in mammals. The molecular pathways of pituitary development in zebra fish are also very similar to the pathways of adenohypophysis (anterior lobe of the pituitary) development in the mouse [77]. Noriega has recently presented important perspectives on the evolution of sex steroids in the vertebrates [4].

Based on histology, the placentae of eutherian mammals are grouped into epitheliochorial (maternal outer epithelium and embryonic placental chorion), endotheliochorial (maternal inner endothelium and embryonic placental chorion), and hemochorial (maternal blood supply and embryonic placental chorion). The epitheliochorial placenta has been considered the least invasive and therefore was thought to be the most primitive of the placental forms (with histotrophic nutrition from uterine "milk"), especially since it is present in the (more primitive) marsupial mammals. The more intimate endotheliochorial and hemochorial placentae (with hemotrophic nutrition) have been viewed as a derived (more advanced) state.

However, recent research has indicated that the noninvasive epitheliochorial placenta is the derived state [78], which formed evolutionarily most recently; it is not clear (yet) which of the two more invasive types of placentation occurred in the last common ancestor of placental mammals [79]. It has also recently been reported that the advanced epitheliochorial placenta evolved at least three times in a convergent manner, and that this form of placenta "is more efficient in nutritional transfer (flow rate by exchange surface)" than the other two placental forms [80].

In fact, using phylogenetic and statistical analyses of molecular and morphological data, Wildman et al. [81]

reported that the ancestral eutherian mammalian placenta had a hemochorial placental surface, a discoid shape, and labyrinth maternofetal interdigitation, with subsequent development and evolution of clade-specific patterns of placentation and convergent evolution within individual eutherian clades. (A clade is a group consisting of a species [extinct or extant] and its descendants, and is the basis for all biological classification in taxonomy.) The selection pressures on the efficiency of placentation (placental morphology) may be due to/from changes in nutritional demand, gestational length, numbers of embryos per pregnancy, uterine shape, and/or maternal body constitution [81].

The most recent evolutionary placental changes appear to involve the macrophage heterogeneity at the maternal–fetal interface (as in the hemochorial placenta of humans), which leads to induction of maternal immune tolerance to fetal antigens (critically important since the conceptus and embryonic portion of the placenta contain both maternally and paternally inherited genetic alleles) and to improved basic homeostatic functions in human pregnancy [31].

ENDOCRINE TOXICITY AND NATIONAL AND INTERNATIONAL REGULATORY ACTIVITIES

BACKGROUND

The crucial roles of the endocrine system in communicating and interacting with other organ systems (and with other organisms), and the vulnerabilities of this essential system have finally been recognized and described. The subsequent and consequent insults from endogenous or exogenous, natural or manmade sources have also begun to be elucidated; we now know that the adverse effects can be to the endocrine system itself, to the endocrine-responsive end organs, to distal organs and organ systems affected by endocrine-responsive organ activities, or to all three.

The “early” publication of *Silent Spring* by Rachel Carson in 1962 [82] told an apocryphal tale of a fictitious small farming community in the midwestern United States, in which one spring, the insects died, then the plants (cultivated and wild) died, then the wild and farm animals died, then the children. As she stated in her introduction, this cascade had never occurred all in one town, but each of these disasters had occurred in one or more towns across the United States. She made the case for indiscriminate overuse of pesticides as the cause, and asked for a ban on the more insidious long-lasting chemicals, specifically dichlorodiphenyltrichloroethane (DDT). She was vilified by pesticide manufacturers and entomologists alike (but early environmentalists agreed with her findings and her warnings). As early as 1963, President Kennedy’s Science Advisory Committee supported her position [83]. In 1969, Congress sent a bill to President Nixon, known as the National Environmental Policy Act (NEPA), which he signed on January 1, 1970, establishing a Council on Environmental Quality (CEQ), which established the U.S. EPA on December 2, 1970. In fact, the EPA can be viewed

as the “extended shadow of Rachel Carson” [84]. Ms. Carson died in 1964 from breast cancer.

Approximately 30 years later, Dr. Theo Colborn, Ms. Dianne Dumoski, and Dr. John Peterson Myers wrote *Our Stolen Future* in 1996 [85] on the adverse effects of pollutants including pesticides and other manmade agents, agricultural runoff, etc., present on the land and in the water, on terrestrial and aquatic plants and animals, including humans. Dr. Colborn’s thesis was confirmed by the continued and growing evidence of oceanic dead zones, contaminated Arctic and Antarctic animals (far distant from the sources of contamination), fouled water supplies, lost crops, deserted farms, wholesale loss of beneficial insects, plants and animals, reduced food supplies, reduced wildlife breeding and survival, etc., with the strong support of the scientific community.

Even before the publication of her book in 1996, Dr. T. Colborn, driven by her concerns for the future, and Dr. C. Clement organized a Work Session in 1991 on Chemically Induced Alterations in Sexual Development: The Human/Wildlife Connection. The introduction read: “Many compounds introduced into the environment by human activity are capable of disrupting the endocrine system of animals, including fish, wildlife and humans. Endocrine disruption can be profound because of the crucial role hormones play in controlling development” [86,87].

Subsequently, Dr. Colborn and a broad range of scientists (at her urging) met at the Ettore Majorana Centre for Scientific Culture, International School of Ethology, in Erice, Sicily in November 1995 and produced a consensus statement reflecting their concern about the effects of synthetic chemicals on brain development. The opening paragraph of that statement reads:

We are certain of the following:

Endocrine-disrupting chemicals can undermine neurological and behavioral development and subsequent potential of individuals exposed in the womb, or in fish, amphibians, reptiles, and birds, the egg. This loss of potential in humans and wildlife is expressed as behavioral and physical abnormalities. It may be expressed as reduced intellectual capacity and social adaptability, as impaired responsiveness to environmental demands, or in a variety of other functional guises. Widespread loss of this nature can change the character of human societies or destabilize wildlife populations. Because profound economic and social consequences emerge from small shifts in functional potential at the population level, it is imperative to monitor levels of contaminants in humans, animals, and the environment that are associated with disruption of the nervous and endocrine systems and reduce their production and release [88].

U.S. GOVERNMENT ACTIVITIES

In 1996, the U.S. Government was convinced and moved rapidly to promulgate Amendments to the Safe Drinking Water Act and the Food Quality Protection Act (FQPA), both in 1996. The FQPA Amendment empowered the U.S. Environmental Protection Agency (EPA) to convene a Federal Advisory Committee, the Endocrine Disruptor Screening and Testing

Advisory Committee (EDSTAC; of which this author was an invited member) which met from 1996 to 1998, and submitted their two-volume report to the U.S. EPA in 1998 (U.S. EPA EDSTAC, 1998) [89]. Their report described an endocrine disruptor as “an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations or subpopulations of organisms, based on scientific principles, data, weight-of-evidence and the precautionary principle” [89]. EDSTAC members could not agree on a definition, hence the “description.” In fact, the inclusion of “the precautionary principle” was the price to get consensus from EDSTAC members on the “description” of an endocrine disruptor.

The EDSTAC report also recommended a two-tiered system to evaluate possible endocrine active chemicals. Tier 1 contained 11 relatively brief assays (screens) to assess the ability of the tested chemicals to interact with the endocrine system (initially estrogen, androgen, and thyroid; E, A, and T), in cells, in culture, in mammals, frogs, and fishes, in vivo and in vitro (to identify hazard; the intrinsic capacity of the chemical to do harm). Tier 2-specific tests for dose response (risk) were recommended for future development and validation.

Also in 1996, an EPA-sponsored workshop was convened to discuss and determine research needs for the risk assessment of health and environmental effects of endocrine disruptors [90].

Very soon after, in 1997, in response to the EPA-sponsored workshop and its recommendations in 1996, an Endocrine Screening Methods Workshop was held in North Carolina (at which this author was present), to develop and enhance methodologies to detect estrogenic and androgenic hormonal and antihormonal activity for chemicals acting via receptor or steroidogenic enzyme mechanisms [91]. The research to develop new and better screens and tests (and end points within the screens and tests) increased rapidly, with academic researchers developing typically short-term assessments with relatively few animals per group, few groups, and new cutting-edge end points (e.g., molecular, embryological, histopathologic, etc.) to discern mechanism(s), and as predictive indicators of subsequent endocrine disruption. But the U.S. EPA and OECD regulators were (and still are) using guideline-compliant studies, with robust numbers of animals per group, at least three treatment groups and a concurrent control group, including long-term multigenerational evaluations, with validated end points, Standard Operating Procedures (SOPs), performed under Good Laboratory Practice (GLP) regulations, to determine the risk. Basic research studies were and are viewed and valued as critical to provide identification and development of new end points to be validated for subsequent use in guideline studies, and to provide important mechanistic information.

The debate between the “academic” scientists and the “regulatory” scientists became public in 2009, with Myers et al. (with 35 coauthors) [92] making the case that the short-term small academic studies should be used in the EPA’s risk

assessments and that the use of GLPs does not guarantee good science [92]. Tyl, who with her staff at RTI International performed many of the guideline, GLP-compliant, multigeneration rodent studies used by the EPA, FDA, and OECD (including reproductive toxicity studies of BPA and 17 β -estradiol in rats and mice; Tyl et al. [93–95]) responded that, in fact, GLP compliance does guarantee good science (although it does not include cutting edge, unvalidated end points), because it guarantees robust group sizes, use of validated end points, appropriate exposures (doses with analytical confirmation of dosed feed, air concentrations or dosing solution concentrations, and appropriate/relevant routes and durations) over generations, complete documentation and retention of all data collected, SOPs, appropriate statistical analyses, formal protocols, any amendments, complete and thorough final reports including summary and individual data tables, and retention of all data, fixed/frozen tissues, slides, etc., for any subsequent reappraisal (“Bisphenol A as a case study” [96]). These guideline studies also are large, lengthy, and therefore expensive, and are typically supported by industry since EPA and OECD require producers, manufacturers, and/or importers to submit these studies for registration of their chemicals under EPA OPPTS (now including just Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], no longer Toxic Substances Control Act [TSCA]), and under OECD. The best approach appears to continue to identify, develop, and validate the best new approaches and end points, etc. In fact, many of the current validated end points in the guideline multigeneration reproductive toxicity studies began as basic research end points which were subsequently validated under GLPs for use in guideline studies, such as neonatal anogenital distance, age at vaginal patency, age at preputial separation (PPS), estrous cyclicity assessments, epididymal sperm numbers, morphology and motility, testicular homogenization-resistant spermatid head counts, ovarian follicle counts, uterine implantation counts, etc. New end points under discussion include examination for retained nipples and/or areolae (on PND 13) and age at testis descent (usually PND 14–21) in prewean male pups, both affected by exposure to antiandrogens, prenatal and postnatal circulating hormone assessments, mammary gland whole mounts in rats of both sexes from PND 4 through adulthood, including during gestation and lactation for females, for quantitative assessment of effects on mammary gland development and differentiation (or involution) after in utero and/or lifetime exposures, initially developed by Dr. S. Fenton EPA/NIEHS [97,98]. Also, new study designs have been developed and are in use by the NTP, such as multiple matings from the same breeding pairs (the Reproductive Assessment by Continuous Breeding [RACB] protocol), and the Modified One-Generation (the MOG) protocol, starting with rat dams exposed during pregnancy and lactation, and the F1 offspring distributed into various groups (or “cassettes”) for developmental, neuro-, immuno-, reproductive, and/or chronic toxicity assessments; one important advantage is that the F1 offspring used in these cassettes were exposed to the test chemical during gestation and lactation. Molecular and histopathologic examination of

target tissues early in development are also being done, with early histopathologic prostatic changes in male fetuses from in utero exposure to estrogenic/antiandrogenic endocrine disruptors being reported, possibly signaling or resulting in increased susceptibility to prostate carcinogenesis in adulthood [99,100], etc., also proposed as predictors of subsequent adult adverse outcomes.

Assessment of effects on steroidogenesis was added later to the Tier 1 assays, when it was recognized that some chemicals (e.g., phthalates [101–109] were potent antiandrogens, but did not act via binding to the AR, but by interference with the earliest steps in steroidogenesis in the mitochondria of the Leydig cells outside the seminiferous tubules in the testes. (Parenthetically, Davis et al. [110] reported that diethylhexyl phthalate [DEHP], an antiandrogenic phthalate, was also antiestrogenic, and Akingbemi et al. [109, p. 780] reported that DEHP was also indirectly estrogenic, because it also increased serum E2 levels, likely from LH induction of aromatase activity in Leydig cells.) Concurrent increases in serum T and E2 levels from phthalate exposure “suggest (to Akingbemi et al. [109]) the possibility of multiple cross talks between androgen, estrogen, and steroid-hormone receptors, indicating that the mechanisms of chemical-induced effects may be more complex than previously thought.” This author views that suggestion as a prescient understatement!

Any test chemical found positive in Tier 1 (based on weight of evidence) would be considered “hazardous,” and would go into Tier 2 testing, which provides tailored tests, including multigenerational dose–response assessment, to identify the “risk” (the probability of an adverse outcome depending on the species, strain, age, sex of the exposed individual, and on the dose, route, timing and duration of exposure) of these chemicals to mammals, birds, amphibians, fish, and invertebrates, the last included since ~95% of the animal biota on earth are invertebrates.

Endocrine effects include direct effects on the traditional endocrine glands, their hormones and receptors, but can also include effects on signaling pathways which interact with many of the body’s systems and processes, such as the reproductive system, embryo–fetal development, the nervous system and behavior, the immune and metabolic systems, the liver, bones, and many other organs and tissues. Goldman and Cooper [111] have shown that toxicants which act centrally (in the brain) can affect the neuroendocrine regulation of reproductive function in female rats; however, neither of the two OECD assay guidelines and none of the 11 U.S. EPA assay guidelines evaluate chemicals for this mechanism of action. It should be noted that Tier 2 tests in whole animals (see later) could also provide evidence of a centrally acting agent.

A list of potential endocrine-disrupting chemicals, first presented by Dr. Colborn et al. [85], has been regularly updated by Dr. Colborn and her staff, with ~870 Endocrine-Disrupting Chemicals on the list from the website of The Endocrine Disruption Exchange (TEDX) as of May 2012. Each chemical in the TEDX list has one or more verified citations to published accessible primary scientific research demonstrating effects

on the endocrine system. This list is maintained and regularly updated on Dr. Colborn’s TEDX website and can be accessed at this website address “<http://www.endocrinedisruption.org>,” and then going to the “ED Program.” There is also a smaller list on the website with oversight by Dr. John Peterson Myers and his staff.

In 1999 (1 year after the release of EDSTAC’s report), the Executive Summary of the National Research Council’s “Committee on Hormonally Active Agents (HAAs) in the Environment” indicated that “Although it is clear that exposures to HAAs at high concentrations can affect wildlife and human health, the extent of the harm caused by exposure to these compounds in concentrations that are common in the environment is debated” (NRC, Committee on HAAs, 1999).

Also in 1999, the National Institutes of Environmental Health Sciences (NIEHS) and other cosponsors held a workshop to characterize the effects from environmental (low) exposures, and to improve risk assessment of endocrine disruptors on human health. One of the six subgroups evaluated the effects of endocrine disruptors on endocrine function during development [22]. This group focused on the regulatory processes in normal development and how exposures to low environmentally relevant doses of endocrine disruptors at critical developmental stages can lead to adverse health effects, including irreversible effects unique to developmental exposures. The group recommended additional research in five areas:

1. Molecular mechanisms of normal development (under epigenetic control)
2. Stage-specific differences in endocrine disruptor effects (for the embryo, fetus, neonate, and adult)
3. Mechanisms of endocrine disruption
4. Dose–response assessment, ranging from environmental doses to doses producing acute toxicity (including nonmonotonic as well as monotonic dose–response curves)
5. Designs of specific screens to accurately predict the unique developmental effects

The group focused, as did EDSTAC, on steroids (estrogens and androgens) and TH, including the critical end points of receptor binding and activation/inhibition, synthesis inhibitors, and plasma transport, and rate of metabolism and clearance. The concern was raised that even measuring “total” concentration of, for example, estradiol and testosterone in fetal plasma will provide misleading results, since the concentration of estrogen-binding plasma proteins (α -fetoprotein) is much higher in fetuses than in adults. As a result, circulating free concentrations of estradiol are ~10-fold lower in fetuses than in adults [112]. Conversely, the fetal levels of free testosterone are very high in fetuses, since rodents do not have a high-affinity plasma-binding protein for testosterone [113]. The group concurred that there is a need for more/better information on hormonal roles in development and for new/better methods to assess chemicals/mixtures for endocrine-disrupting activity, particularly during critical periods

of organogenesis, including improvements in the designs of multigeneration reproductive toxicity studies [22]. Knobil and other specialists in endocrine disruptors also expressed concerns about HAAs in the environment [114].

In contrast to the earlier concerns about low environmentally relevant doses, Juberg [115] wrote a review on the evaluation of endocrine modulators (his preferred term to endocrine disruptors) and implications for human health. He indicated that although high doses of these modulators are likely to cause adverse effects, the available (in 2000) “laboratory, wildlife, and epidemiological data do not provide consistent or convincing evidence that industrial chemicals and/or environmental pollutants, suspected of modulating estrogenic pathways, are related to adverse health effects in humans” [115].

Ten years after the publication of “*Our Stolen Future*,” London [116] also posted his views that the threats presented in the book from manmade chemicals in the environment have not (yet) surfaced and are overstated and underproved. Part of the problem is that many aspects of published endocrine disruptor activities are very different from the standard model of “traditional toxicology.” They include reported non-monotonic dose–response curves, and qualitatively different responses at high versus low doses of the same compound. In addition, researchers are reporting that endocrine-disrupting chemicals violate the almost axiomatic assumption that there is some level of exposure, the threshold, below which smaller amounts of contaminant have no effect. London [116] and others have reported data which indicate that there is no threshold of effect for an endocrine-disrupting compound when it is added to a hormone system that is already active, etc.

OECD TEST GUIDELINES

In the fall of 2007 (9 years after the EDSTAC report), the international Organisation for Economic Cooperation and Development (OECD, in which the United States is a member), released one Guideline for the Testing of Chemicals:

- No. 440, adopted on October 16, 2007: Uterotrophic Bioassay in Rodents: A Short-Term Screening Test for Oestrogenic Properties (21 pp.) and a second guideline in 2009 (Table 12.2)
- No. 441, adopted on September 7, 2009: Hershberger Bioassay in Rats: A Short-Term Screening Assay for (Anti)-Androgenic Properties (20 pp.). These two assays were standardized and validated by an international group of university, industrial, governmental and contract laboratories, using known and unknown (to the testing laboratories) chemicals selected by OECD for their endocrine activities/mechanism(s) of action (see Table 12.2). Descriptions of these two assays follow:

The *Uterotrophic Assay* (2007) relies for its sensitivity on an animal test system in which the hypothalamic–pituitary–ovarian axis is not functional, so circulating endogenous estrogen levels are low. This results in very low uterine

weights and maximum range of possible responses. The postwean/prepubertal female and the postpubertal ovariectomized female both satisfy this requirement. The prepubertal females (10 per group, with at least two dose groups and a vehicle control) are dosed once daily (by oral gavage or subcutaneous injection) for three consecutive days (with body weights and clinical observations recorded daily); the ovariectomized females are typically dosed from 3 to 10 days, once daily (again with at least two dose groups and a vehicle control). Both models are necropsied 24 h after the last dose, with terminal body weights and uterine weights (fresh and/or blotted) recorded. A group with a positive control (a known potent estrogen) may also be used to provide maximum uterine weight stimulation, and both an estrogen agonist and an antagonist may also be used to detect antiestrogenic activity (less than maximal estrogen-dependent uterine growth in the presence of an agonist and an antagonist). If the study is done correctly and the test chemical is estrogenic, the uterine weights (wet and blotted), absolute and relative to terminal body weight, will exhibit dose-related statistically significant increases [117].

The *Hershberger Bioassay* (2009) functions as a mechanistic in vivo assay to detect androgen agonists, androgen antagonists, and 5 α -reductase inhibitors. Castrated peripubertal males (10 per group) are dosed once daily (by oral gavage or subcutaneous injection) for 10 consecutive days. The animals are terminated ~24 h after the last dose. The terminal body weight and weights of five target androgen-dependent tissues

1. Ventral prostate
2. Seminal vesicles plus fluids and coagulating glands
3. Levator ani-bulbocavernosus muscle
4. Paired Cowper’s glands
5. Glans penis

are recorded. A known androgenic compound may be used to provide maximal stimulation of androgen-sensitive organ growth (i.e., a positive control) (Sloan et al. [118]); a positive androgen and an antiandrogen may also be used to detect antiandrogenic activity (resulting in less than maximal testosterone-dependent organ weight gain in the presence of an agonist and an antagonist) [119]. O’Connor et al. [120] also evaluated a 15 day intact male rat assay for detecting antiandrogens. An immature intact stimulated weanling version of this assay to detect antiandrogens was suggested when these assays were first being evaluated [121], but it was not promulgated as an acceptable alternative OECD bioassay. (See text on the U.S. EPA Hershberger Bioassay, No. 890.1400, listed later for the explanation.) It is not known whether additional OECD guidelines will be forthcoming.

U.S. EPA TEST GUIDELINES

In October 2009, the U.S. EPA, Office of Prevention, Pesticides and Toxic Substances (OPPTS), publicly released its Series 890 OPPTS Harmonized Test Guidelines: Endocrine Disruptor Screening Program Test Guidelines

(see Table 12.2), after U.S. EPA Scientific Advisory Panel Review of the EDSP Proposed Tier 1 Screening Battery in 2008. (An earlier overview of the U.S. EPA Tier 1 Screening Battery is presented by Eldridge and Laws [132].) The EPA Series included the following 11 Validated Final Screening Test Guidelines (Table 12.2):

890.1100 Amphibian Metamorphosis (Frog) (50 pp., 710 K)

This screening assay is designed to identify substances which interfere with the HPT axis. Since amphibian metamorphosis (from water-dwelling tadpole to terrestrial frog) is a thyroid-dependent process, this assay is viewed as a generalized vertebrate model to evaluate conserved structures and functions of the HPT axis. The species of choice is *Xenopus laevis*. Twenty tadpoles per group (tank) at stage 51 (when there is no evidence yet of metamorphosis) are exposed to one of three different concentrations of the test chemical (at a minimum) and a dilution water control (and a solvent control if necessary) in a flow-through system, for 21 days, with four replicates. The end points are daily mortality checks, checks for abnormal swimming behavior, malformations, lesions, etc., Days 7 and 21 (terminal) hind limb length, snout to vent length (SVL), developmental stage, terminal body wet weight, and thyroid gland histology (5 Tadpoles/dose from each replicate tank). If the test chemical is thyroid-active, there should be evidence of accelerated development (including metamorphosis), asynchronous development, and/or histological changes in the thyroid gland; antithyroid activity is evidenced by delayed development (no metamorphosis), although nonthyroidal general toxicity can also cause delays, stunted growth, and abnormal development (see also Kloas and Lutz [122]). An alternative 15 day Intact Adult Male Rat Screening Assay has also been presented to evaluate antithyroid chemicals [119].

890.1150 AR Binding (Rat Prostate Cytosol) (34 pp., 485 K)

This assay determines the ability of the test compound to compete with tritiated (H3) ligand (R1881 Solution) for binding in rat ventral prostate homogenate. The ligand and test chemical are prepared in the same solvent: 100% ethanol, water, or dimethyl sulfoxide (DMSO). A standard curve is recommended with five dilutions from 1×10^{-5} to 3×10^{-10} . Triamcinolone acetate is used to prevent the binding of R1881 to any progesterone receptors in the cytosolic preparation. Adult male rats (90 days of age) are castrated; the ventral prostate is collected as close to 24 h after castration as possible, trimmed, weighed, and centrifuged. The low-salt prostate cytosol supernatant contains the cytosolic receptor. The supernatants are pooled from all rats; protein content is determined; and saturation radioligand assays are conducted to assure that the AR is present in adequate amounts and functioning with the appropriate affinity. The actual assay involves tritiated R1881, triamcinolone acetate, Radioinert R1881, and diluted prostatic cytosol.

The results are calculated as total, nonspecific, and specific H3-R1881 binding in nM. It is typically graphically presented as a saturation curve and a Scatchard plot; the IC_{50} (inhibition concentration at 50% inhibition) is estimated and the % relative binding affinity (RBA) is calculated. There are specific performance criteria in the assay description.

890.1200 Aromatase (Human Recombinant) (24 pp., 205 K)

This screening assay is intended to identify chemicals that affect the endocrine system (e.g., steriodogenesis) by inhibiting the activity of aromatase (CYP19, the enzyme which converts androgens to estrogens in multiple target tissues in all vertebrates), through an interaction of the test chemical with the substrate-binding site on the enzyme. The assay measures the conversion of androgen to estrogen in microsomes isolated from various target tissues containing aromatase, or in cell lines with recombinant aromatase, and cytochrome P450 reductase. A radioactive substrate (H3-androstenedione) and NADPH are added to microsomes containing the aromatase (CYP19) and reductase complex. Tritiated water ($3H_2O$) is released into the culture medium during the conversion of androstenedione to estrone, and can be quantified in the medium as a direct measurement of aromatase activity per unit reaction time. Competitive inhibition of aromatase activity by test chemicals can be detected by serial reaction tubes containing increasing concentrations of the test chemical.

890.1250 ER-Binding Assay Using Rat Uterine Cytosol (ER-RUC) (61 pp., 627 K)

This assay is designed to detect whether a compound can interact with the ERs isolated from rat uterus. This assay will not detect whether the interaction is specifically one-site competitive binding, and will not characterize the precise strength of the binding; it is therefore not considered appropriate for use in quantitative structure-activity relationship (SAR) model development for estrogen binding. As with the other Tier 1 assays, it is to be used as part of the screening program, including other assays, to detect substances that can interact with the estrogen hormonal system; it also does not mean that when the substance is positive, that it will necessarily cause adverse effects in humans or ecological systems. This assay measures the ability of a radiolabeled ligand (tritiated 17β -estradiol) to interact with the ER in the presence of increasing concentrations of a test chemical. Rat uterine cytosol is incubated in test tubes with increasing concentrations of a test chemical, and an aliquot of radiolabeled (H3)-estradiol. If the test substance interacts with the receptor's hormone-binding domain, less radioligand can bind, so the presence of increasing amounts of an active competitor results in a descending H3-estradiol dose-response curve. This curve does NOT differentiate between an agonist (acts like an endogenous estrogen after binding) and an antagonist (acts like an estrogen-binding inhibitor after binding). Compounds which do not displace the radioligand are considered inactive for estrogen binding.

**890.1300 ER Transcriptional Activation
(Human Cell line [HeLa-9903]), (23 pp., 336 K)**

In vitro transcriptional activation (TA) assays are based on the production of a reporter gene product induced by exposure to the test chemical, following binding of the chemical to a specific receptor and subsequent downstream TA. TA assays have been used to evaluate specific gene expression regulated by specific nuclear receptors such as the ERs; therefore, they are now included for use to detect chemicals which act by binding to the human ER α receptor resulting in estrogen transactivation regulated by the ER. This assay is designed to evaluate the ability of the test chemical to function as an ER α ligand and activate an agonist response; it also provides mechanistic information. (It is important to note that although this process is considered one of the key endocrine disruption mechanisms to trigger adverse health effects, other important mechanisms do exist and include actions mediated via other nuclear receptors linked to the endocrine system, and interactions with the steroidogenic enzymes such as metabolic activation or deactivation of hormones; distribution of hormones to target tissues; and clearance of hormones from the body.) This assay detects (human) ER α -mediated TA in the stably transfected hER α -HeLa-9903 cell line from ER α -mediated activation of the firefly luciferase reporter gene. This produces increased expression of luciferase which, in turn, converts luciferin (in the mixture) to a bioluminescent product. The resulting detectable chemiluminescence is then measured. This assay and the AR-binding transactivational assays are also used to identify environmental endocrine disruptors in wildlife [123].

Note: In the *Federal Register* (Vol. 77, No. 162, Tuesday, August 21, 2012), Federal Agency Response to Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Recommendations on the Usefulness and Limitations of the Lumi-Cell[®] ER (BGILuc ER TA [Estrogen Receptor Transcriptional Activation]) Test Method, An In Vitro Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals: The U.S. EPA responded that “they regard the BGILuc ER TA test method as an alternative to the current test method currently used in their Endocrine Disruptor Screening Program” (<http://iccvam.niehs.nih.gov/SppDocs/FedDocs/FR.2012.20549htm>, accessed in 2012). The original (and current) U.S. EPA Test Guideline for ER TA is No. 890.1300 (see earlier): Estrogen Receptor Transcriptional Activation (with Human Cell line HeLa-9903).

**890.1350 Fish Short-Term Reproduction
Assay (93 pp., 15 MB)**

This a short-term reproduction assay with the fathead minnow (*Pimephales promelas*) is designed to evaluate reproductive fitness as an integrated measure of toxicant effects, and measures a number of histological and biochemical

end points that indicates adverse effects to the HPG axis. Newly sexually mature spawning male and female fish of the same age are initially exposed to a 14 day pretrial control period to confirm readiness (production of at least 15 eggs/female/day/replicate tank). They are then exposed for 21 days, 4 females and 2 males per tank, by randomized block design, with four replicates, and four doses of the test chemical, plus a dilution control and a solvent control if necessary (for a total of 6 tanks per replicate). The fish are weighed at the start of the study, and fed frozen brine shrimp twice daily. Biological end points include fecundity, fertilization success, adult survival, secondary sexual characteristics, vitellogenin (VTG), analyzed from blood samples from both sexes at termination, found predominantly in females, at 10 to 100 times the expected VTG levels in males, with increased levels associated with egg production in females; the increased presence of VTG in males indicates that the test chemical has estrogenic/antiandrogenic activity, egg counts in females (and any eggs in the testes; or ovotestis), plasma sex steroids (optional), gonadosomal index (GSI, the ratio of the weight of the gonads to the body weight of the fish), and gonadal weight and histology. See also an overview of the U.S. EPA tests for endocrine-disrupting chemicals in aquatic vertebrates [124], in aquatic ecosystems [125], and in other wildlife [126]. Soon after, Iwanowicz and Blazer reported estrogen-associated endocrine disruption in fish, specifically effects on reproductive and immune physiology [127].

890.1400 Hershberger Bioassay (Rat) (24 pp., 214 K)

The OECD initiated a high priority activity in 1998 to revise existing guidelines and to develop new guidelines for the screening and testing of potential endocrine disruptors, including development of a Test Guideline for the Rat Hershberger Bioassay, used by the pharmaceutical industry for decades, and first standardized in 1962 [128], and extensively validated in 2001–2008. The OECD Test Guideline 441, released in 2009, was the outcome of the OECD validation test program (see earlier) and is the basis of this 2009 EPA OPPTS Test Guideline. In this EPA test guideline, the castrated peripubertal males are also exposed to graded doses of the test chemical (10/group) or to a vehicle control (and to a known androgen, or a known androgen and a known antiandrogen) by oral gavage or subcutaneous injection once daily for 10 days. The animals are also terminated 24 h after the last dose, and accessory sex organs are removed, trimmed in, and weighed. This EPA test guideline is exactly the same as the OECD 441 (see earlier). Due to animal welfare concerns about the use of surgical castration, the intact (uncastrated) stimulated weanling male was also evaluated by EPA (as well as by OECD). However, in both the OECD and EPA validation processes, the intact weanling version of the Hershberger Bioassay did not consistently detect effects on androgen-dependent organ weights from exposure to weak antiandrogens at the doses tested (OECD, 2008). “Therefore, it was not included in this

Test Guideline” (OPPTS 890.1400, p. 3). See also Yamada [129] for more discussion on this assay.

890.1450 Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats (31 pp., 1.4 M)

The purpose of this test guideline is to detect chemicals and/or mixtures that interact with the endocrine system by identifying effects on pubertal development and thyroid function in the intact juvenile/pubertal female rat. This assay can detect antithyroid, estrogenic, or antiestrogenic chemicals (which act via changes in receptor binding or in steroidogenesis), or chemicals which alter pubertal development via changes in LH, FSH, PRL, or GH levels, or by alterations in hypothalamic function. The guideline recommends that the performing laboratory breeds in-house females for the same mate/GD 0 date or purchases pregnant rats to litter on the same day, and retains offspring females to obtain 15 females per group (body weight means and variance terms equivalent across groups, and no siblings in the same group) at weaning on postnatal day (PND) 21, with a minimum of two dosed groups and a vehicle control group. The females are housed 2–3 per cage and exposures must be initiated no later than PND 22. Beginning on PND 22, the test chemical is administered by oral gavage once daily, with daily body weights and clinical observations. Beginning on PND 22, the females are also examined daily for vaginal patency (opening); once vaginal patency has occurred (typically on PND 31–35 in Sprague-Dawley rats), daily vaginal smears are collected to determine the start of estrous cycling, and the subsequent presence and duration of the stages from PND 22 through PND 42. (These dosing, timing, duration, and assessment parameters will detect estrogenic chemicals, pubertal delay, and antithyroid effects.) The females are terminated on PND 42, within 2 h after the last dose. At necropsy, trunk blood is collected, centrifuged, and serum retained for hormone and blood chemistry assessments. The ovaries (paired), uterus, thyroid (with parathyroids and attached portion of the trachea), liver, kidneys (paired), pituitary, and adrenal glands (paired) are removed, weighed (paired organs are weighed as pairs), and fixed in 10% buffered neutral formalin for subsequent histopathology (detailed instructions are presented). Hormonal assays include T4 (tetraiodothyronine), and TSH by radioimmunoassay (RIA), immunoradiometric assay (IRMA), enzyme-linked immunosorbent assay (ELISA), or time-resolved immunofluorescent procedures; again detailed instructions are provided in the test guideline. Blood chemistry includes measurement of creatinine and blood urea nitrogen (BUN). Histopathology is performed on uterus, thyroid, one ovary, and one kidney (presumably from each female but this is not specified, nor is the total number of females/group to be so evaluated) and evaluated for pathological abnormalities and potential treatment-related effects.

890.1500 Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats (28 pp., 1.3 MB)

This is essentially the same assay as the Pubertal Assay in females (except that it is in males). The purpose of this assay in pubertal males is to detect chemical substances or mixtures that interact with the endocrine system, by identifying effects on pubertal development and thyroid function in the intact pubertal male. This assay can detect chemicals with antithyroid, androgenic, or antiandrogenic activity (mediated by AR, or steroid enzyme-mediated), or agents which alter pubertal development via changes in GTHs, PRL, or hypothalamic functions. The guideline recommends purchasing pregnant dams or breeding in-house females with the same mate date (GD 0), to get male offspring born on the same day, placing 15 per group (mean body weights and variance terms equivalent across groups and no siblings in the same group) at weaning on PND 21, with a minimum of two dosed groups and a vehicle control group. Dosing is once daily by oral gavage from PNDs 23 to 53. Body weights and clinical observations are recorded daily. Beginning on PND 30, the males are examined daily, at approximately the same time, for PPS, the ability to retract the glans penis from the penile shaft; the date of complete PPS is recorded. Males are killed on PND 53 and trunk blood taken immediately, centrifuged, and serum stored at -20°C or colder for subsequent hormone assays and blood chemistry; hormones include T4, TSH, and testosterone; blood chemistry assessments include creatinine and BUN. At necropsy, the paired testes, paired epididymides, ventral prostate, dorsolateral prostate, seminal vesicles with coagulating glands and fluid, levator ani plus bulbocavernosus muscles, thyroid gland (with attached portion of trachea), liver, paired kidneys, pituitary, and paired adrenal glands are removed, weighed (paired organs weighed as pairs), and retained in fixative for subsequent histopathology (again detailed instructions are included in the test guideline). Testes and epididymides are fixed in Bouin's fixative (or modified Davidson's fixative); the other organs are fixed in 10% buffered neutral formalin. One testis, one epididymis, the thyroid, and one kidney (presumably from each male, but not so specified, nor is the number of males/group to be so evaluated) are examined for pathological abnormalities and potential treatment-related effects.

An overview of the EPA EDSP pubertal assays as a screen in both males and females is presented by Stoker and Zorrilla [130]. As previously mentioned, an alternative 15 day intact Adult Male Rat Screening Assay, to evaluate antithyroid chemicals, was presented by Becker et al. [119].

Note: Testis descent (into the scrotal sacs) is also an androgen-dependent postnatal landmark, occurring typically on PND 14–21 in Sprague-Dawley rats (or up to PND 28 depending on offspring and/or treatment variables). This end point can be assessed if the dams and their pups are reared in-house (prior to weaning on PND 21), and is very sensitive

to antiandrogen exposures in utero and/or early in lactation; it is not currently part of any of the EPA Tier 1 assays.

890.1550 Steroidogenesis (Human Cell Line H295R) (45 pp., 502 K)

This protocol is designed to identify xenobiotics that affect the steroidogenic pathway beginning with the sequence of reactions occurring after the production of the two GTH hormone receptors (FSHR and LHR), through the production of testosterone and estradiol-estrone. However, this assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland. The protocol describes obtaining the H295R human cell line cells from frozen stock, and culturing the cells for a minimum of four additional passages prior to their use in this assay, plating of the H295R cells (ATCC CLR-2128) in a 24-well plate setup and exposure of the cells to test chemicals. The cells are cultured in Nu serum (analyzed prior to use for the presence of testosterone and estradiol). The procedure is to expose the cells in the medium with the test chemical and assay for production of hormones; one useful caveat is to test each test chemical before initiation of the actual exposure experiments for potential interference with the hormone measurement system to be utilized; this is especially true for antibody-based assays such as ELISAs and RIAs, because some chemicals are known to interfere with these tests [131]. Performance criteria include

1. Method detection limit (testosterone: 100 pg/mL and estradiol: 10 pg/mL)
2. Spike sample recovery (supplemented medium spiked with at least two concentrations of each

hormone of interest; example, T: 500 and 2500 pg/mL; E2: 50 and 250 pg/mL; when analyzed, the average recovery rates [on triplicate measures] should not deviate more than 30% from nominal concentrations)

3. Hormone cross-reactivity: No significant (less than or equal to 30% of basal hormone production of the respective hormone) cross-reactivity with any of the other hormones produced by the cells is expected to occur. These include cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17 α -pregnenolone, 17 α -progesterone, deoxycortisol, cortisol, DHEA, androstenedione, and estrone

890.1600 Uterotrophic Assay (Rat) (24 pp., 272 K)

This Test Guideline is based on the OECD Uterotrophic Test Guideline No. 440. The only difference between this OPPTS Test Guideline (TG) and the OECD TG 440 is the expressed preference in this EPA OPPTS test guideline for using the ovariectomized female rat and the subcutaneous injection dosing route, which reflect the specific role of this assay in the EDSP Tier 1 Battery of Assays.

Note: There are only two OECD test guidelines to date, the Uterotrophic Assay and the Hershberger Bioassay, and there are 11 Tier 1 assays in the U.S. EPA OPPTS Test Guideline list, so the EPA is able to assign specific roles to individual Tier 1 assays.

Please note also that a review of endocrine toxicology was published in 2010 including discussion of the Tier 1 assays and mammalian and wildlife effects [132].

TABLE 12.2

International Validated Tier 1 Assays for Endocrine Disruptors

No.	Title	Date Adopted
OECD		
440	Uterotrophic Bioassay in Rodents: A Short-Term Screening Test for Oestrogenic Properties	October 16, 2007
441	Hershberger Bioassay in Rats: A Short-Term Screening Assay for (Anti)-Androgenic Properties	September 7, 2009
U.S. EPA		
890.1100	Amphibian Metamorphosis (Frog)	October 2009
890.1150	Androgen Receptor Binding (Rat Prostate Cytosol)	October 2009
890.1200	Aromatase (Human Recombinant)	October 2009
890.1250	Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC)	October 2009
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903])	October 2009
890.1350	Fish Short-Term Reproduction Assay	October 2009
890.1400	Hershberger Bioassay (Rat)	October 2009
890.1450	Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats	October 2009
890.1500	Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats	October 2009
890.1550	Steroidogenesis (Human Cell Line-H295R)	October 2009
890.1600	Uterotrophic Assay (Rat)	October 2009

CONCERNS

Two recurring concerns about endocrine disruptors are the presence of low-dose effects and nonmonotonic dose–response curves. The debate began over a decade ago with whether or not there actually were low-dose effects, and whether or not there actually were nonmonotonic dose-responsive effects. Two recent important publications in 2012 have presented the arguments and data (from academic publications) for the presence of low-dose effects and for nonmonotonic dose–response curves in both human and animal studies on endocrine-disrupting chemicals, with nonlinear responses observed at different levels of biological complexity, and including the recognition of and increasing evidence for the combination of competing monotonic responses to result in an overall nonmonotonic dose–response curve [133]. They concluded that effects at low doses cannot be predicted by effects observed at high doses for these endocrine-disrupting chemicals, which included industrial chemicals, plastic components and plasticizers, pesticides, phytoestrogens, preservatives, surfactants and sunblock, among others [133]. Dr. L. Birnbaum, Director of the NIEHS and NTP, earlier this year, specifically mentioned the Vandenberg et al.'s paper in an editorial in *Environmental Health Perspectives* (EHP), and made a strong case for the need (even stronger, the requirement) to develop more sophisticated study designs over a broad range of doses (including environmentally relevant doses) to better support regulatory decision making to protect human health [134].

Also more recently, Dr. Birnbaum in another editorial in EHP [135] announced NIEHS's New Strategic Plan to pursue some of the "big influences" which have been "understudied," which interact with more traditional environmental exposures, example, the microbiome, inflammation pathways, immunologic pathways, nutrition and epigenetic processes, to define the "exposome," the totality of the exposures encountered by humans. The NTP is leading the Tox21 initiative along with NIH's National Center for Comparative Genomics, the U.S. EPA and the U.S. FDA; this high-throughput testing program is expected to be faster, and uses less animals, but also "shows great promise for moving toxicology into a predictive science." NIEHS will evaluate mixtures, effects of exposure throughout the lifespan, including prenatal exposures, and how they may link to adult disease (related to the Barker hypothesis on in utero food deprivation and adult consequences). Also, Dr. Birnbaum states that "the antiquated idea that the dose makes the poison is overly simplistic" and that biology is affected by low doses of chemicals, often within the range of general population exposure (i.e., environmental exposures), and that the biological changes in response to these exposures may be harmful, especially during certain stages of development. Therefore, she believes that low dose research must go "hand-in-hand" with the lifespan approach. She concludes that the NIEHS will also help translate the nation's research investments into public health intervention, new policy, and preventive clinical practice.

An additional (third) concern, by this author, is the performance, publication, and use of studies with exposures of the test

chemical only to adult animals (even over long term) for assessment of endocrine effects on reproductive (and/or other) systems (e.g., [136]). Most of the known endocrine disruptors are active only when exposures occur in utero and/or in early postnatal/prewean developmental stages when the systems of concern are developing and maturing. It is therefore reasonable to assume that most (all?) of the yet unknown endocrine disruptors may also exhibit the same age-specific activities and the developing organ systems may exhibit the same age-specific sensitivities, so that new endocrine disruptors will likely not be detected by adult-only exposure studies (or at best only at much higher doses, likely confounded by systemic toxicity).

A fourth concern, voiced by Dr. Bernard Weiss [137], is that not only sexually dimorphic reproductive structures, functions, or behaviors are affected by developmental exposures to endocrine disruptors. Measures of cognitive and other sexually dimorphic behaviors, not necessarily or specifically related to reproduction, are also affected by developmental exposures to endocrine disruptors acting as developmental neurotoxins. One example is that developmental exposure to polychlorinated biphenyls or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in strikingly different behavioral responses in males and females. Such differences may be affected by cultural and/or biological differences, but there are sexual dimorphisms in brain structure, including the sexually dimorphic size of the medial preoptic nucleus (the sexually dimorphic nucleus in the preoptic area; SDN-POA) of the hypothalamus which is larger in male rats and mice, and in male humans, than in females. Cortical lateralization of the SDN-POA in humans also appears to be sex linked, because, as in rodents, boys/men show more asymmetry than girls/women in this brain region [137].

There is also a fifth concern: The requirements for the use of the EPA and OECD test guidelines in whole animals for submission of chemical registration dossiers for their use in the United States and Europe (e.g., REACH [European Community Regulation: Registration, Evaluation, Authorization, and Restriction of Chemical substances; released on June 1, 2007]) and the call for new and better animal studies (see earlier) are resulting (and expected to further result) in increased and increasing animal usage worldwide, increasing costs for whole animal testing, increased time to complete the whole animal tests, not enough competent, experienced testing laboratories/facilities/space and technical staff to perform the required screening and testing in a timely manner, along with increasing numbers of chemicals to evaluate. Current estimates are 6000–9000 chemicals for U.S. EPA alone, and over 80,000 chemicals worldwide (and 2000 new ones introduced yearly) to which humans are potentially exposed but whose toxicity remains largely unknown (Mardas Daneshian, CAAT-EU, 05/02/2012). But the concerns are not just scientific or fiscal. There are also strong international movements to decrease/ban whole animal testing, with strong Animal Rights and Animal Welfare groups (including more militant groups such as PETA: People for the Ethical Treatment of Animals) espousing ethical perspectives and (emotional) concerns, especially, but not exclusively, for mammals including nonhuman primates.

In fact, the European Union has decreed whole animal testing bans for cosmetic ingredients in Europe in 2013, whether or not there are suitable replacement or substitute tests available (7th Amendment to the Cosmetics Directive, Animal Welfare Directive, 2009). A note of caution: The species of test animal, the strain of rat/mouse, even the vendor of the “same strains,” and/or the presence or type of enrichment present in the cage can result in distinct differences in adrenocortical or other responses to a given stimulus [138], and by extrapolation, to distinct differences in responses of other end points of interest to the same exposures (chemical, dose, route, timing, and duration).

Concurrent with these concerns about whole animal testing, there has been tremendous progress in the development of new high-throughput, high content in vitro and in silico testing, and bioinformatics science and technology.

The U.S. National Research Council of the National Academy of Sciences published a book in 2007 entitled: *Toxicity Testing in the 21st Century: A Vision and a Strategy*. It challenged the current, traditional approach (for the earlier reasons) and suggested an alternative approach with strong emphasis on in vitro and in silico evaluations to identify the molecular, genetic (and epigenetic) signatures of test chemicals to determine their hazard and ultimately their risk to target species, including humans, using a weight-of-evidence approach.

U.S. EPA ToxCast™ SYSTEM

The U.S. EPA rapidly responded [139] by announcing the establishment of their ToxCast™ Research Program, to develop, validate, and employ predictive, high-throughput cell-based assays (many of human origin) to evaluate perturbations of universal key pathways of toxicity, and to conduct targeted testing against those pathways. Mapping the entirety of these pathways (the “Human Toxome Project”) would be a huge undertaking (on the order of the Human Genome Project), but it is viewed as working with the European REACH initiative which includes the testing ban on cosmetics, pesticide regulation, and endocrine disruptor screening, while reducing animal suffering (M. Daneshian, CAAT-EU, Center for Alternatives to Animal Testing-European Union, University of Konstanz, Konstanz, Germany, email dated May 02, 2012, 7:15 a.m.).

In March 2011, Dr. Robert Kavlock of the U.S. EPA presented a video transcript IdeaScape Tutorial Webinar on EPA Chemical Safety for Sustainability, discussing, in summary form, the draft framework and progress of an integrated EPA research program, which, he indicated, “encompasses computational toxicology, nanotechnology, endocrine-disrupting chemicals,” as well as development of “new prediction techniques, innovative technologies for chemical toxicity testing, and design of tools to advance the management of chemical risks.”

The EPA’s perspective is that the ToxCast Research Program, as developed by EPA’s National Center for Computational Toxicology (NCCT) of EPA’s Office of Research and Development (ORD), will develop cost-effective

innovative approaches to rapidly screen and prioritize many chemicals for further toxicological testing (at least initially). Using data from state-of-the-art high-throughput screening (HTS) assays, based on the assays developed and used by the pharmaceutical industry for decades to prioritize compounds for further development, ToxCast is building computational models to better predict the potential toxicity of chemicals (i.e., the hazard), and to provide data to the Agency to set priorities for more targeted toxicological evaluations (including dose, route, duration, timing, etc., i.e., the risk). This will allow EPA to target those chemicals and effects possessing the greatest potential for adverse outcomes for further assessment.

ToxCast is a multiyear effort divided into three phases:

Phase I: Proof of Concept: 300 chemicals (pesticides) of known toxicity from submissions of specified animal and other tests (see later) to EPA FIFRA for registration are being tested by U.S. EPA (and their partners, collaborators, and contractors) in the over 500 different HTS bioassays, example, very recent publications/presentations from the U.S. EPA and collaborators: Kleinstreuer and coworkers [140], Sipes et al. [141], Ellis-Hutchings et al. [140]; from industry: Daston [142]; from European scientists: Piersma [143], Robinson et al. [144]; and from Canadian scientists: Bahn and Hales [145]. The results allow creation of a predictive signature for each toxic effect (this phase is complete)

Phase II: Confirmation and Expansion: EPA (and their partners and contractors) will generate HTS data on at least 300 additional chemicals for comparison with, and confirmation of the Phase I results (this phase is in progress).

Phase III: Expansion of HTS testing to thousands of environmental chemicals (i.e., chemicals in the environment) and pesticides, for which little toxicological information is currently available; results of the HTS bioassays will be used to predict the toxicity of these chemicals.

The whole animal toxicity data are available to EPA FIFRA for their use because the EPA has been requiring manufacturers for years to provide data on a pesticide to be registered for manufacture and use, to show that its intended use does not cause “unreasonable adverse effects on the environment,” including unacceptable risks to humans, the environment (animals and plants), or large adverse economic effects. Social and environmental costs and benefits of the use of any pesticide are also taken into account by EPA. Therefore, manufacturers must provide data to EPA from these many different animal study types evaluating potential hazard and risk (exposure) to register their product for use. These include rat 28 and 90 day toxicity studies, rat chronic/2 year carcinogenicity bioassays, mouse 18 month carcinogenicity bioassays, rat multigenerational reproductive toxicity studies, and rodent (rat or mouse) and rabbit prenatal developmental toxicity studies, as well as information on other mammalian toxicity effects (e.g., neurotoxicity, immunotoxicity, metabolism), and environmental assessments, such as studies on aquatic life, wildlife and plant toxicity, pesticide use patterns,

environmental fate and persistence, and pesticide residue identification and environmental levels.

ToxCast is therefore also developing a toxicology reference database containing the data from all of these animal toxicity studies, called "ToxRefDB," a joint effort between EPA's NCCT and EPA's Office of Pesticide Programs (OPP). However, at this time, ToxRefDB contains only hazard information with no exposure information currently included (and therefore it does not yet assess risk). ToxCast is obviously under intensive and extensive development. According to the EPA, when it is completed, the HTS information will include physical, chemical, and predicted biological activities based on existing structure-activity models, biochemical properties based on the HTS assays, cell-based phenotypic assays, and genomic and metabolomics analyses of cells by contractors, collaborators within EPA, with the National Toxicology Program and the National Institutes of Health Chemical Genomics Center. This multidimensional dataset will require appropriate computational models (also under development) for integrating the various chemical, biological (including genetic and epigenetic), and toxicological data into profiles and models predicting toxicity [139]. It will be used, initially, to prioritize the subsequent *in vivo* animal testing of products which have limited toxicity data available, such as antimicrobial pesticides, inert ingredients in pesticide products, manufacturing process impurities, metabolites and environmental degradation products of concern, and existing and new industrial chemicals.

But the ultimate role of ToxCast, once the three phases have been completed and all the new assays validated, according to presentations by and discussions with involved EPA scientists, is to entirely replace the whole animal testing component so that the *in vitro* and *in silico* assays will determine potential hazard and (with exposure information) potential risk for chemicals for the U.S. EPA (and ultimately the world?).

The need for and the strengths of the proposed *in vitro*/*in silico* assessments of universal molecular pathways involved in toxicity (i.e., ToxCast) are obvious. However, the existing and proposed evaluations in ToxCast do not adequately address (or assess) the following concerns (in this author's opinion).

The determination of risk requires knowledge of dose, route, timing, and duration of exposure to the organism of interest, none of which will be determined from *in vitro* and/or *in silico* assessments. How will risk (versus hazard) be determined? Will a new risk paradigm (need to) be developed?

For reproductive, developmental, and endocrine toxicity, one or both parents is/are exposed to the test chemical and proceed to subject it to the adult ADME (Absorption, Distribution, Metabolism, and Elimination/Excretion) prior to transport to the egg/sperm or conceptus. What the spermatozoa in the male reproductive tract are exposed to may be very different from what the adult father was exposed to; what the egg in the ovary is exposed to may also be very different from what the mother was exposed to. The fertilized egg developing into a conceptus

in utero (or in a shelled egg) is also likely exposed to something different from what the egg/sperm, and the mother/father were exposed to. The ToxCast Program does not currently assess the metabolic consequences when the initial chemical agent is changed/altered/metabolized *in vivo*; it may be detoxified or toxified as it moves through the adult male or female, and in the case of a pregnant mammal, also through the maternal placenta and into the fertilized egg or conceptus, or from maternal blood to maternal milk into the postnatal offspring (depending on the stage of development).

The predictability of the EPA EDSP screens and tests and the ToxCast assays for risk to the target(s) of interest, example, environmental organisms, including vertebrates (including humans), and invertebrates, is of paramount importance; that is the point of the assessments! When the screens and tests use the actual organisms of interest as in the EPA EDS Program (e.g., mammals, birds, frogs, fish, invertebrates) then the predictability is strong; if a surrogate is related to the organism of interest (i.e., a rat or mouse to represent the human mammal), the predictability is still there, but less strong. When the assays are *in vitro* or *in silico*, the predictability absolutely depends on the fidelity of the assay to mimic how the actual organism(s) of interest handles the chemical, not just the source of the cells or enzymes used in the assay, but, for risk assessment, the *in vivo* context. It is understood that the pathways to be evaluated in the *in vitro*/*in silico* assays are considered to be universal and highly conserved. However, a process or mechanism must be developed to determine what the effect would be on the test chemical from adult/parental ADME, placental ADME, offspring ADME, etc., from the organisms of interest (at vulnerable stages) acting on the test chemical, as well as to assess and predict the effect of the test chemical on the organisms of interest (again, at vulnerable stages). The nature or extent of the effect as it happens in a whole organism in its environment will likely be very different from how it happens in cells in a Petri dish, or in computer simulations.

CONCLUSIONS

Our knowledge and concern about the endocrine system and endocrine toxicity have increased enormously since the publication of *Silent Spring* in 1962. With growing worldwide scientific information and scientific champions such as Drs. T. Colborn (the Endocrine Disruption Exchange), L.E. Gray, Jr. and R. Kavlock (EPA), P.M.D. Foster (NTP), R.T. Zoeller (U. Mass., Amherst), G. Daston (Procter & Gamble Co.), F. vom Saal (U. Missouri, Columbia) and many others, national and international governmental regulatory agencies have moved to establish and validate screens and tests to identify which manmade and natural chemicals/compounds/mixtures result in hazard (Tier 1), and (more importantly, at least to this author) which chemicals/compounds/mixtures (at which doses, routes, timing and durations of exposure) result in risk (Tier 2) to terrestrial and aquatic wildlife, domesticated animals, and humans. The more we understand about the mechanisms of endocrine activity and disruption, the more complicated (and lengthy and expensive) the current *in vitro*/*in vivo* research/

testing screens and in vivo tests have become. Worldwide, we do not have the capacity (competent laboratories and staff) to perform the screens and tests well and quickly; we are ethically and emotionally uncomfortable about the current huge and increasing use of animals for our screens and tests (not just primates or even just mammals). And we are overwhelmed by the current and projected costs. The development of the U.S. EPA ToxCast program to use in vitro and in silico models and informatics, cell-based assays, genomic and metabolomics analyses, assessment of universal relevant molecular pathways, etc. holds great promise to detect hazardous chemicals; the conditions under which the chemicals result in risk will be much more difficult to determine. We are not there yet. The things we do not now know (or do not now think important) may be as or more important, in the long run, than the things we know and value now. Only time and continued great international effort (by regulatory and academic scientists alike) will tell.

REFERENCES

1. Sower, S.A., M. Freamat, and S.I. Kavanaugh, The origins of the vertebrate hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) endocrine systems: New insights from lampreys. *Gen Comp Endocrinol*, 2009. 161(1): 20–29.
2. Baker, M.E., Evolution of adrenal and sex steroid action in vertebrates: A ligand-based mechanism for complexity. *Bioessays*, 2003. 25(4): 396–400.
3. Gilbert, S.F., Cell interactions at a distance: Hormones as mediators of development, amphibian metamorphosis. *Developmental Biology*, Fifth edition, 1997. Sunderland, MA: Sinauer Associates, Inc., Chapter 19.
4. Noriega, N., Evolutionary perspectives on sex steroids in the vertebrates, sex steroids. Scott M.K., ed., 2012. ISBN: 978-953-307-857-1, InTech. Available from: <http://www.intechopen.com/books/sex-steroids/evolutionary-perspectives-on-sex-steroids-in-the-vertebrates>, pp. 1–34.
5. Ozen, S. and S. Darcan, Effects of environmental endocrine disruptors on pubertal development. *J Clin Res Pediatr Endocrinol*, 2011. 3(1): 1–6.
6. Henderson, I.W., Endocrine system in vertebrates, 2001. DOI: 10.1038/ngp.els.0001845.
7. Goodyear, C.G., L. De Stephano, W.H. Lai, H.J. Guyda, and B.I. Posner, Characterization of insulin-like growth factor receptors in rat anterior pituitary, hypothalamus, and brain. *Endocrinology*, 1984. 114(4): 1187–1195.
8. Gregory, S.J. and U.B. Kaiser, Regulation of gonadotropins by inhibin and activin. *Semin Reprod Med*, 2004. 22(3): 253–267.
9. Hinuma, S., Y. Habata, R. Fujii, Y. Kawamata, M. Hosoya, S. Fukusumi, C. Kitada, Y. Masuo, T. Asano, H. Matsumoto, M. Sekiguchi, T. Kurokawa, O. Nishimura, H. Onda, and M. Fujino, A prolactin-releasing peptide in the brain. *Nature*, 1998. 393(6682): 272–276.
10. Milnes, M.R., Jr., R.N. Roberts, and L.J. Guillelte, Jr., Effects of incubation temperature and estrogen exposure on aromatase activity in the brain and gonads of embryonic alligators. *Environ Health Perspect*, 2002. 110(Suppl 3): 393–396.
11. Orlando, E.F., W.P. Davis, and L.J. Guillelte, Jr., Aromatase activity in the ovary and brain of the eastern mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent. *Environ Health Perspect*, 2002. 110(Suppl 3): 429–433.
12. Petraglia, F., P. Sacerdote, A. Cossarizza, S. Angioni, A.D. Genazzani, C. Franceschi, M. Muscettola, and G. Grasso, Inhibin and activin modulate human monocyte chemotaxis and human lymphocyte interferon-gamma production. *J Clin Endocrinol Metab*, 1991. 72(2): 496–502.
13. Xia, Y. and A.L. Schneyer, The biology of activin: Recent advances in structure, regulation and function. *J Endocrinol*, 2009. 202(1): 1–12.
14. Chen, Y.G., Q. Wang, S.L. Lin, C.D. Chang, J. Chuang, and S.Y. Ying, Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. *Exp Biol Med (Maywood)*, 2006. 231(5): 534–544.
15. Sulyok, S., M. Wankell, C. Alzheimer, and S. Werner, Activin: An important regulator of wound repair, fibrosis, and neuroprotection. *Mol Cell Endocrinol*, 2004. 225(1–2): 127–132.
16. Ying, S.Y., Inhibins and activins: Chemical properties and biological activity. *Proc Soc Exp Biol Med*, 1987. 186(3): 253–264.
17. Burger, H.G. and M. Igarashi, Inhibin: Definition and nomenclature, including related substances. *J Clin Endocrinol Metab*, 1988. 66(4): 885–886.
18. Zhao, E., H. Hu, and V.L. Trudeau, Secretoneurin as a hormone regulator in the pituitary. *Regul Pept*, 2010. 165(1): 117–122.
19. Doerge, D.R. and D.M. Sheehan, Goitrogenic and estrogenic activity of soy isoflavones. *Environ Health Perspect*, 2002. 110(Suppl 3): 349–353.
20. Tatsumi, K., K. Miyai, T. Notomi, K. Kaibe, N. Amino, Y. Mizuno, and H. Kohno, Cretinism with combined hormone deficiency caused by a mutation in the *PIT1* gene. *Nat Genet*, 1992. 1(1): 56–58.
21. Howdeshell, K.L., A model of the development of the brain as a construct of the thyroid system. *Environ Health Perspect*, 2002. 110(Suppl 3): 337–348.
22. Bigsby, R., R.E. Chapin, G.P. Daston, B.J. Davis, J. Gorski, L.E. Gray, K.L. Howdeshell, R.T. Zoeller, and F.S. vom Saal, Evaluating the effects of endocrine disruptors on endocrine function during development. *Environ Health Perspect*, 1999. 107(Suppl 4): 613–618.
23. Cockburn, A., Evolutionary ecology of the immune system: Why does the thymus involute? *Funct Ecol*, 1992. 6(3): 364–370.
24. Upadhyay, S. and L. Zamboni, Preliminary observations on the role of the mesonephros in the development of the adrenal cortex. *Anat Rec*, 1982. 202(1): 105–111.
25. Pohorecky, L.A. and R.J. Wurtman, Adrenocortical control of epinephrine synthesis. *Pharmacol Rev*, 1971. 23(1): 1–35.
26. Satoh, M., Histogenesis and organogenesis of the gonad in human embryos. *J Anat*, 1991. 177: 85–107.
27. Hotchkiss, A.K., J.S. Ostby, J.G. Vandenberg, and L.E. Gray, Jr., Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague-Dawley rat. *Environ Health Perspect*, 2002. 110(Suppl 3): 435–439.
28. Sawyer, H.R., P. Smith, D.A. Heath, J.L. Juengel, S.J. Wakefield, and K.P. McNatty, Formation of ovarian follicles during fetal development in sheep. *Biol Reprod*, 2002. 66(4): 1134–1150.
29. Zamboni, L., J. Bezaud, and P. Mauleon, The role of the mesonephros in the development of the sheep fetal ovary. *Ann Biol Anim Biochim Biophys*, 1979. 19(4B): 1153–1178.
30. Sadler, T.W., *Langman's Medical Embryology*, Fifth edition, 1985. Philadelphia, PA: Williams & Wilkins.

31. Houser, B.L., Decidual macrophages and their roles at the maternal–fetal interface. *Yale J Biol Med*, 2012. 85(1): 105–118.
32. Dela, C.R., A.F. Suffredini, and J.D.e.a. Page, Activation of the kallikrein-kinin system after endotoxin administration to normal human volunteers. *Blood*, 1993. 81: 3313–3317.
33. McLean, P.G., M. Perretti, and A. Ahluwalia, Kinin B(1) receptors and the cardiovascular system: Regulation of expression and function. *Cardiovasc Res*, 2000. 48(2): 194–210.
34. Steppan, C.M., S.T. Bailey, S. Bhat, E.J. Brown, R.R. Banerjee, C.M. Wright, H.R. Patel, R.S. Ahima, and M.A. Lazar, The hormone resistin links obesity to diabetes. *Nature*, 2001. 409(6818): 307–312.
35. Lee, J.H., J.L. Chan, N. Yiannakouris, M. Kontogianni, E. Estrada, R. Seip, C. Orlova, and C.S. Mantzoros, Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: Cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. *J Clin Endocrinol Metab*, 2003. 88(10): 4848–4856.
36. Heilbronn, L.K., J. Rood, L. Janderova, J.B. Albu, D.E. Kelley, E. Ravussin, and S.R. Smith, Relationship between serum resistin concentrations and insulin resistance in non-obese, obese, and obese diabetic subjects. *J Clin Endocrinol Metab*, 2004. 89(4): 1844–1848.
37. Campbell, R.K., N. Satoh, and B.M. Degnan, Piecing together evolution of the vertebrate endocrine system. *Trends Genet*, 2004. 20(8): 359–366.
38. Holland, L.Z., R. Albalat, K. Azumi, E. Benito-Gutierrez, M.J. Blow, M. Bronner-Fraser, F. Brunet, T. Butts, S. Candiani, L.J. Dishaw, D.E. Ferrier, J. Garcia-Fernandez, J.J. Gibson-Brown, C. Gissi, A. Godzik, F. Hallbook, D. Hirose, K. Hosomichi, T. Ikuta, H. Inoko, M. Kasahara, J. Kasamatsu, T. Kawashima, A. Kimura, M. Kobayashi, Z. Kozmik, K. Kubokawa, V. Laudet, G.W. Litman, A.C. McHardy, D. Meulemans, M. Nonaka, R.P. Olinski, Z. Pancer, L.A. Pennacchio, M. Pestarino, J.P. Rast, I. Rigoutsos, M. Robinson-Rechavi, G. Roch, H. Saiga, Y. Sasakura, M. Satake, Y. Satou, M. Schubert, N. Sherwood, T. Shiina, N. Takatori, J. Tello, P. Vopalensky, S. Wada, A. Xu, Y. Ye, K. Yoshida, F. Yoshizaki, J.K. Yu, Q. Zhang, C.M. Zmasek, P.J. de Jong, K. Osoegawa, N.H. Putnam, D.S. Rokhsar, N. Satoh, and P.W. Holland, The amphioxus genome illuminates vertebrate origins and cephalochordate biology. *Genome Res*, 2008. 18(7): 1100–1111.
39. Nakabayashi, K., H. Matsumi, A. Bhalla, J. Bae, S. Mosselman, S.Y. Hsu, and A.J. Hsueh, Thyrostimulin: A heterodimer of two new human glycoprotein hormone subunits, activates the thyroid-stimulating hormone receptor. *J Clin Invest*, 2002. 109: 1445–1452.
40. Sudo, S., Y. Kuwabara, J.I. Park, S.Y. Hsu, and A.J. Hsueh, Heterodimeric fly glycoprotein hormone- $\alpha 2$ (GPA2) and glycoprotein hormone-beta5 (GPB5) activate fly leucine-rich repeat-containing G protein-coupled receptor-1 (DLGR1) and stimulation of human thyrotropin receptors by chimeric fly GPA2 and human GPB5. *Endocrinology*, 2005. 146(8): 3596–3604.
41. Kubokawa, K., Y. Tando, and S. Roy, Evolution of the reproductive endocrine system in chordates. *Integr Comp Biol*, 2010. 50(1): 53–62.
42. Bertrand, S., F.G. Brunet, H. Escriva, G. Parmentier, V. Laudet, and M. Robinson-Rechavi, Evolutionary genomics of nuclear receptors: From twenty-five ancestral genes to derived endocrine systems. *Mol Biol Evol*, 2004. 21(10): 1923–1937.
43. Uchida, K., S. Moriyama, H. Chiba, T. Shimotani, K. Honda, M. Miki, A. Takahashi, S.A. Sower, and M. Nozaki, Evolutionary origin of a functional gonadotropin in the pituitary of the most primitive vertebrate, hagfish. *Proc Natl Acad Sci U S A*, 2010. 107(36): 15832–15837.
44. Nozaki, M., Y. Oshima, M. Miki, T. Shimotani, H. Kawauchi, and S.A. Sower, Distribution of immunoreactive adenohypophyseal cell types in the pituitaries of the Atlantic and the Pacific hagfish, *Myxine glutinosa* and *Eptatretus burgeri*. *Gen Comp Endocrinol*, 2005. 143(2): 142–150.
45. Dore, R.M. and A.J. Baron, Evolution of POMC: Origin, phylogeny, posttranslational processing, and the melanocortins. *Ann NY Acad Sci*, 2011. 1220: 34–48.
46. Freamat, M. and S.A. Sower, Glycoprotein hormone receptors in the sea lamprey *Petromyzon marinus*. *Zool Sci*, 2008. 25(10): 1037–1044.
47. Freamat, M. and S.A. Sower, A sea lamprey glycoprotein hormone receptor similar with gnathostome thyrotropin hormone receptor. *J Mol Endocrinol*, 2008. 41(4): 219–228.
48. Roch, G.J., E.R. Busby, and N.M. Sherwood, Evolution of GnRH: Diving deeper. *Gen Comp Endocrinol*, 2011. 171(1): 1–16.
49. Dos Santos, S., S. Mazan, B. Venkatesh, J. Cohen-Tannoudji, and B. Querat, Emergence and evolution of the glycoprotein hormone and neurotrophin gene families in vertebrates. *BMC Evol Biol*, 2011. 11: 332.
50. Cole, L.A., New discoveries on the biology and detection of human chorionic gonadotropin. *Reprod Biol Endocrinol*, 2009. 7: 8.
51. Holland, C.A. and J.N. Dumont, Oogenesis in *Xenopus laevis* (Daudin). IV. Effects of gonadotropin, estrogen and starvation on endocytosis in developing oocytes. *Cell Tissue Res*, 1975. 162(2): 177–184.
52. Targonska, K. and D. Kucharczyk, The application of hCG, CPH and Ovopel in successful artificial reproduction of goldfish (*Carassius auratus auratus*) under controlled conditions. *Reprod Domest Anim*, 2011. 46(4): 651–655.
53. Arslan, M., J. Lobo, A.A. Zaidi, and M.H. Qazi, Effect of mammalian gonadotropins (HCG and PMSG) on testicular androgen production in the spiny-tailed lizard, *Uromastix hardwicki*. *Gen Comp Endocrinol*, 1977. 33(1): 160–162.
54. Heinig, J.A., F.W. Keeley, P. Robson, S.A. Sower, and J.H. Youson, The appearance of proopiomelanocortin early in vertebrate evolution: Cloning and sequencing of POMC from a Lamprey pituitary cDNA library. *Gen Comp Endocrinol*, 1995. 99(2): 137–144.
55. Gorbman, A. and S.A. Sower, Evolution of the role of GnRH in animal (Metazoan) biology. *Gen Comp Endocrinol*, 2003. 134(3): 207–213.
56. Wu, S., L. Page, and N.M. Sherwood, A role for GnRH in early brain regionalization and eye development in zebrafish. *Mol Cell Endocrinol*, 2006. 257–258: 47–64.
57. Tello, J.A. and N.M. Sherwood, Amphioxus: Beginning of vertebrate and end of invertebrate type GnRH receptor lineage. *Endocrinology*, 2009. 150(6): 2847–2856.
58. Tello, J.A., S. Wu, J.E. Rivier, and N.M. Sherwood, Four functional GnRH receptors in zebrafish: Analysis of structure, signaling, synteny and phylogeny. *Integr Comp Biol*, 2008. 48(5): 570–587.
59. Oakley, A.E., D.K. Clifton, and R.A. Steiner, Kisspeptin signaling in the brain. *Endocr Rev*, 2009. 30(6): 713–743.
60. Kitahashi, T., S. Ogawa, and I.S. Parhar, Cloning and expression of kiss2 in the zebrafish and medaka. *Endocrinology*, 2009. 150(2): 821–831.

61. Kanda, S. and Y. Oka, Evolutionary insights into the steroid sensitive kiss1 and kiss2 neurons in the vertebrate brain. *Front Endocrinol (Lausanne)*, 2012. 3: 28.
62. Mano, H. and Y. Fukada, A median third eye: Pineal gland retraces evolution of vertebrate photoreceptive organs. *Photochem Photobiol*, 2007. 83(1): 11–18.
63. Strassman, R., Part I: The building blocks; Chapter 4. *The Psychedelic Pineal. DMT: The Spirit Molecule*, 2001. Rochester, VT: Park Street Press, pp. 81–82.
64. Venturi, S., F.M. Donati, A. Venturi, and M. Venturi, Environmental iodine deficiency: A challenge to the evolution of terrestrial life? *Thyroid*, 2000. 10(8): 727–729.
65. Kupper, F.C., L.J. Carpenter, G.B. McFiggans, C.J. Palmer, T.J. Waite, E.M. Boneberg, S. Woitsch, M. Weiller, R. Abela, D. Grolimund, P. Potin, A. Butler, G.W. Luther, 3rd, P.M. Kroneck, W. Meyer-Klaucke, and M.C. Feiters, Iodide accumulation provides kelp with an inorganic antioxidant impacting atmospheric chemistry. *Proc Natl Acad Sci USA*, 2008. 105(19): 6954–6958.
66. Okabe, M. and A. Graham, The origin of the parathyroid gland. *Proc Natl Acad Sci USA*, 2004. 101(51): 17716–17719.
67. Slipka, J., V. Pospisilova, and J. Slipka, Jr., Evolution, development and involution of the thymus. *Folia Microbiol (Praha)*, 1998. 43(5): 527–530.
68. Willey, A., *Amphioxus and the Ancestry of the Vertebrates*, Columbia University Biological Series III1894. New York: MacMillan.
69. Slipka, J., Lymphonic cells invade the thymus from hemopoietic tissue during the 3rd month post conception. *J Craniofacial Genet Dev Biol*, 1986. (Suppl 2): 267–276.
70. Pieler, T. and Y. Chen, Forgotten and novel aspects in pancreas development. *Biol Cell*, 2006. 98(2): 79–88.
71. Yui, R. and T. Fujita, Immunocytochemical studies on the pancreatic islets of the ratfish *Chimaera monstrosa*. *Arch Histol Jpn*, 1986. 49(3): 369–377.
72. Jones, I.C., J.G. Phillips, and D. Bellamy, The adrenal cortex throughout the vertebrates. *Br Med Bull*, 1962. 18: 110–114.
73. Milano, E.G. and F. Accardi, Evolutionary trends in adrenal gland of anurans and urodeles. *J Morphol*, 1986. 189(3): 249–259.
74. McGuire, N.L. and G.E. Bentley, Neuropeptides in the gonads: From evolution to pharmacology. *Front Pharmacol*, 2010. 1: 114.
75. Lockwood, G.M., W.L. Ledger, D.H. Barlow, N.P. Groome, and S. Muttukrishna, Measurement of inhibin and activin in early human pregnancy: Demonstration of fetoplacental origin and role in prediction of early-pregnancy outcome. *Biol Reprod*, 1997. 57(6): 1490–1494.
76. Poon, S.K., W.K. So, X. Yu, L. Liu, and W. Ge, Characterization of inhibin alpha subunit (inha) in the zebrafish: Evidence for a potential feedback loop between the pituitary and ovary. *Reproduction*, 2009. 138(4): 709–719.
77. Pogoda, H.M. and M. Hammerschmidt, How to make a teleost adenohypophysis: Molecular pathways of pituitary development in zebrafish. *Mol Cell Endocrinol*, 2009. 312(1–2): 2–13.
78. Carter, A.M. and A. Mess, Evolution of the placenta in eutherian mammals. *Placenta*, 2007. 28(4): 259–262.
79. Mess, A. and A.M. Carter, Evolution of the placenta during the early radiation of placental mammals. *Comp Biochem Physiol A Mol Integr Physiol*, 2007. 148(4): 769–779.
80. Vogel, P., The current molecular phylogeny of eutherian mammals challenges previous interpretations of placental evolution. *Placenta*, 2005. 26(8–9): 591–596.
81. Wildman, D.E., C. Chen, O. Erez, L.I. Grossman, M. Goodman, and R. Romero, Evolution of the mammalian placenta revealed by phylogenetic analysis. *Proc Natl Acad Sci USA*, 2006. 103(9): 3203–3208.
82. Carson, R., *Silent Spring*, 1962. Boston, MA: Houghton Mifflin.
83. Graham, F.J., Labour EPA. Rachel Carson. *EPA J*, 1978. (November/December): 1–4.
84. Lewis, J., About EPA. The birth of EPA. *EPA J*, 1985. (November): 1–6.
85. Colborn, T.D., D. Dumsoski, and J.P. Myers, *Our Stolen Future*, 1996. New York: Dutton.
86. Colborn, T. and C. Clement, Chemically-induced alterations in sexual and functional development: The wildlife/human connection. *Advances in Modern Environmental Toxicology*, Vol. XXI, 1992. Princeton, NJ: Princeton Scientific Pub. Co., pp. 1–8.
87. Colborn, T., The wildlife/human connection: Modernizing risk decisions. *Environ Health Perspect*, 1994. 102(Suppl 12): 55–59.
88. Colborn, T., Impact of endocrine disruptors on brain development and behavior. Preface. *Environ Health Perspect*, 2002. 110(Suppl 3): 335.
89. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. USEPA, August, 1998.
90. Kavlock, R.J., G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaattari, G. Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheehan, T. Sinks, and H.A. Tilson, Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *Environ Health Perspect*, 1996. 104(Suppl 4): 715–740.
91. Gray, L.E., Jr., W.R. Kelce, T. Wiese, R. Tyl, K. Gaido, J. Cook, G. Klinefelter, D. Desaulniers, E. Wilson, T. Zacharewski, C. Waller, P. Foster, J. Laskey, J. Reel, J. Giesy, S. Laws, J. McLachlan, W. Breslin, R. Cooper, R. Di Giulio, R. Johnson, R. Purdy, E. Mihaich, S. Safe, T. Colborn, Endocrine Screening Methods Workshop Report: Detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. *Reprod Toxicol*, 1997. 11(5): 719–750.
92. Myers, J.P., F.S. vom Saal, B.T. Akingbemi, K. Arizono, S. Belcher, T. Colborn, I. Chahoud, D.A. Crain, F. Farabollini, L.J. Guillette, Jr., T. Hassold, S.M. Ho, P.A. Hunt, T. Iguchi, S. Jobling, J. Kanno, H. Laufer, M. Marcus, J.A. McLachlan, A. Nadal, J. Oehlmann, N. Olea, P. Palanza, S. Parmigiani, B.S. Rubin, G. Schoenfelder, C. Sonnenschein, A.M. Soto, C.E. Talsness, J.A. Taylor, L.N. Vandenberg, J.G. Vandenbergh, S. Vogel, C.S. Watson, W.V. Welshons, and R.T. Zoeller, Why public health agencies cannot depend on Good Laboratory Practices as a criterion for selecting data: The case of bisphenol A. *Environ Health Perspect*, 2009. 117(3): 309–315.
93. Tyl, R.W., C.B. Myers, M.C. Marr, B.F. Thomas, A.R. Keimowitz, D.R. Brine, M.M. Veselica, P.A. Fail, T.Y. Chang, J.C. Seely, R.L. Joiner, J.H. Butala, S.S. Dimond, S.Z. Cagen, R.N. Shiotsuka, G.D. Stropp, and J.M. Waechter, Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. *Toxicol Sci*, 2002. 68(1): 121–146.
94. Tyl, R.W., C.B. Myers, M.C. Marr, C.S. Sloan, N.P. Castillo, M.M. Veselica, J.C. Seely, S.S. Dimond, J.P. Van Miller, R.N. Shiotsuka, D. Beyer, S.G. Hentges, and J.M. Waechter, Jr., Two-generation reproductive toxicity study of dietary bisphenol A in CD-1 (Swiss) mice. *Toxicol Sci*, 2008. 104(2): 362–384.
95. Tyl, R.W., C.B. Myers, M.C. Marr, C.S. Sloan, N.P. Castillo, M.M. Veselica, J.C. Seely, S.S. Dimond, J.P. Van Miller, R.S. Shiotsuka, G.D. Stropp, J.M. Waechter, Jr., and S.G. Hentges,

- Two-generation reproductive toxicity evaluation of dietary 17beta-estradiol (E2; CAS No. 50-28-2) in CD-1 (Swiss) mice. *Toxicol Sci*, 2008. 102(2): 392–412.
96. Tyl, R.W., Basic exploratory research versus guideline-compliant studies used for hazard evaluation and risk assessment: Bisphenol A as a case study. *Environ Health Perspect*, 2009. 117(11): 1644–1651.
 97. Fenton, S., NIEHS, *Presentation: Mammary Gland Whole Mount Round Robin* at Health Canada, Ottawa, Ontario, Canada; December 4, 2009.
 98. Fenton, S.E., Early life environmental exposures: Lifelong impact in mammary gland development and function. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): Symposium Abstract S200.
 99. Ho, S.M., W.Y. Tang, J. Belmonte de Frausto, and G.S. Prins, Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. *Cancer Res*, 2006. 66(11): 5624–5632.
 100. Prins, G.S., W.Y. Tang, J. Belmonte, and S.M. Ho, Developmental exposure to bisphenol A increases prostate cancer susceptibility in adult rats: Epigenetic mode of action is implicated. *Fertil Steril*, 2008. 89(2 Suppl): e41.
 101. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-octyl phthalate. *Reprod Toxicol*, 2002. 16(5): 721–734.
 102. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-hexyl phthalate. *Reprod Toxicol*, 2002. 16(5): 709–719.
 103. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di-isononyl phthalate. *Reprod Toxicol*, 2002. 16(5): 679–708.
 104. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di-isodecyl phthalate. *Reprod Toxicol*, 2002. 16(5): 655–678.
 105. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. *Reprod Toxicol*, 2002. 16(5): 529–653.
 106. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-butyl phthalate. *Reprod Toxicol*, 2002. 16(5): 489–527.
 107. Kavlock, R., K. Boekelheide, R. Chapin, M. Cunningham, E. Faustman, P. Foster, M. Golub, R. Henderson, I. Hinberg, R. Little, J. Seed, K. Shea, S. Tabacova, R. Tyl, P. Williams, and T. Zacharewski, NTP Center for the Evaluation of Risks to Human Reproduction: Phthalates expert panel report on the reproductive and developmental toxicity of butyl benzyl phthalate. *Reprod Toxicol*, 2002. 16(5): 453–487.
 108. Tyl, R.W., C.B. Myers, M.C. Marr, P.A. Fail, J.C. Seely, D.R. Brine, R.A. Barter, and J.H. Butala, Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. *Reprod Toxicol*, 2004. 18(2): 241–264.
 109. Akingbemi, B.T., R. Ge, G.R. Klinefelter, B.R. Zirkin, and M.P. Hardy, Phthalate-induced Leydig cell hyperplasia is associated with multiple endocrine disturbances. *Proc Natl Acad Sci USA*, 2004. 101(3): 775–780.
 110. Davis, B.J., R.R. Maronpot, and J.J. Heindel, Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol Appl Pharmacol*, 1994. 128(2): 216–223.
 111. Goldman, J.M. and R.L. Cooper, The Impact of centrally acting pesticidal/environmental toxicants on the neuroendocrine regulation of reproductive function in the female rodent: Relevance to human reproductive risk assessment. *Target Organ Toxicology Series*, 3rd Edn., A.W.T. Hayes and J.A. Gardner, eds., 2010. London, U.K: Informa Healthcare Publishers.
 112. Montano, M.M., W.V. Welshons, and F.S. vom Saal, Free estradiol in serum and brain uptake of estradiol during fetal and neonatal sexual differentiation in female rats. *Biol Reprod*, 1995. 53(5): 1198–1207.
 113. Danzo, B.J. and B.C. Eller, The ontogeny of biologically active androgen-binding protein in rat plasma, testis, and epididymis. *Endocrinology*, 1985. 117(4): 1380–1388.
 114. Knobil, E., H.A. Bern, J. Burger, M. Fry, J. Giesy, J. Gorski, C.J. Grossman, L.J. Guillette, Jr., B.S. Hulka, I.J.R. Lamb, L.A., S. Safe, A. Soto, J.J. Stegeman, S.H. Swann, and F.S. Vom Saal, *Hormonally Active Agents in the Environment*, 1999. Washington, DC: National Academy Press.
 115. Juber, D.R., An evaluation of endocrine modulators: Implications for human health. *Ecotoxicol Environ Saf*, 2000. 45(2): 93–105.
 116. London, W.M., “Our Stolen Future.” Ten Years Later American Council on Environmental Health (ACSH), 2006. Posted May 31, 2006. http://www.acsh.org/healthissues/new-sid.1345/healthissue_detail.asp, pp. 1–5.
 117. Tyl, R.W., M.C. Marr, S.S. Brown, E.A. Dolbow, and C.B. Myers, Validation of the intact rat weanling uterotrophic assay with notes on the formulation and analysis of the positive control chemical in vehicle. *J Appl Toxicol*, 2010. 30(7): 694–698.
 118. Sloan, C.S., K.D. Vick, N.M. Kuney, P.A. Fail, R.A. Becker, and R.W. Tyl, GLP-compliant evaluation and standardization of the peripubertal castrate male rat Hershberger assay for oral exposure of test agents. *Reprod Toxicol*, 2013. 35: 108–116.
 119. Becker, R.A., D.R. Bergfelt, S. Borghoff, J.P. Davis, B.T. Hamby, J.C. O'Connor, A.M. Kaplan, C.S. Sloan, R.W. Tyl, M. Wade, and M.S. Marty, Interlaboratory study comparison of the 15-day intact adult male rat screening assay: Evaluation of an antithyroid chemical and a negative control chemical. *Birth Defects Research*, 2011. 95: 63–78.
 120. O'Connor, J.C., S.R. Frame, and G.S. Ladics, Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens. *Toxicol Sci*, 2002. 69(1): 92–108.

121. Ashby, J., P.A. Lefevre, H. Tinwell, J. Odum, and W. Owens, Testosterone-stimulated weanlings as an alternative to castrated male rats in the Hershberger anti-androgen assay. *Regul Toxicol Pharmacol*, 2004. 39(2): 229–238.
122. Kloas, W. and I. Lutz, *The Hypothalamic–Pituitary–Gonadal Axis in Anuran Amphibians*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
123. Brown, T.R., *Androgen Receptor Binding and Transactivation Assays Identify Environmental Chemicals as Endocrine Disruptors*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
124. Ankley, G.T., S.J. Degitz, M.L. Haasch, K.M. Jensen, R.D. Johnson, A.W. Olmstead, J.E. Tietge, and G. Cripe, *Overview of USEPA Tests with Aquatic Vertebrates for Detecting and Assessing Endocrine Disrupting Chemicals*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
125. Jordan, S.J., W.H. Benson, C.M. Foran, E.R. Bennett, and E.M. Snyder, *Endocrine-Disrupting Compounds in Aquatic Ecosystems*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
126. LeBlanc, G.A., *Overview of Endocrine Disruptor Ecotoxicity in Wildlife*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
127. Iwanowicz, L.R. and V.S. Blazer, An overview of estrogen-associated endocrine disruption in fishes: Evidence of effects on reproductive and immune physiology, pp. 266–275. In RC Cipriano, A Buckner, and IS Shchelkunov, eds., *Aquatic Animal Health, A continuing dialog between Russia and the United States. Proceedings of the third bilateral conference between the United States and Russia: Aquatic Animal Health*, Shepherdstown, WV, July 12–20. Michigan State University, East Lansing, MI, 2009.
128. Dorfman, R.I., *Standard Methods Adopted by Official Organization*, 1962. New York: Academic Press.
129. Yamada, T., *Male Reproductive Endpoints and the Rodent Hershberger Assay*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
130. Stoker, T.E. and L.M. Zorrilla, *The Effects of Endocrine Disrupting Chemicals on Pubertal Development in the Rat: Use of the EDSP Pubertal Assays as a Screen*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
131. Shapiro, R.P. and L.B. Page, Interference by 2,3-dimercapto-1-propanol (BAL) in angiotensin/radioimmuno assay. *J Lab Clin Med*, 1976. 88(2): 222–231.
132. Eldridge, J.C. and S.C. Laws, *The U.S. EPA's Tier 1 Screening Battery for Endocrine Disruptor Compounds*, Third edition. Target Organ Toxicology Series, A.W. Hayes, J.A. Thomas, and D.E. Gardner, eds., Vol. 27, 2010. London, U.K: Informa Healthcare Publishers.
133. Vandenberg, L.N., T. Colborn, T.B. Hayes, J.J. Heindel, D.R. Jacobs, Jr., D.H. Lee, T. Shioda, A.M. Soto, F.S. Vom Saal, W.V. Welshons, R.T. Zoeller, and J.P. Myers, Hormones and endocrine-disrupting chemicals: Low-dose effects and non-monotonic dose responses. *Endocr Rev*, 2012. 33(3): 378–455.
134. Birnbaum, L.S., Environmental chemicals: Evaluating low-dose effects. *Environ Health Perspect*, 2012. 120(4): A143–A144.
135. Birnbaum, L.S., NIEHS's new strategic plan. *Environ Health Perspect*, 2012. 120(8): 298.
136. Ernest, S.R., M.G. Wade, C. Lalancette, Y.Q. Ma, R.G. Berger, B. Robaire, and B.F. Hales, Effects of chronic exposure to an environmentally relevant mixture of brominated flame retardants on the reproductive and thyroid system in adult male rats. *Toxicol Sci*, 2012. 127(2): 496–507.
137. Weiss, B., Sexually dimorphic nonreproductive behaviors as indicators of endocrine disruption. *Environ Health Perspect*, 2002. 110(Suppl 3): 387–391.
138. Turnbull, A.V. and C.L. Rivier, Sprague-Dawley rats obtained from different vendors exhibit distinct adrenocorticotropin responses to inflammatory stimuli. *Neuroendocrinology*, 1999. 70(3): 186–195.
139. Dix, D.J., K.A. Houck, M.T. Martin, A.M. Richard, R.W. Setzer, and R.J. Kavlock, The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol Sci*, 2007. 95(1): 5–12.
140. Ellis-Hutchings, R.G., R.S. Settivari, A.T. McCoy, N.C. Kleinstreuer, V.A. Marshall, T.B. Knudsen, and E.W. Carney, Linking ToxCast™ Signatures with functional consequences: Proof of concept study using known inhibitors of vascular development. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): Platform Abstract 41.
141. Sipes, N.S., M.T. Martin, and T. Knudsen, Species-specific predictive signatures of developmental toxicity using the ToxCast™ Chemical Library. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): ILSI-HESI Symposium Abstract S23.
142. Daston, G.P., Predictive developmental toxicity: Profiles and modes of action. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): ILSI-HESI Symposium Abstract S22.
143. Piersma, A.H., Approaches for predicting developmental toxicity: An integrated testing strategy. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): Symposium Abstract S25.
144. Robinson, J.F., E.C. Tonk, A. Verhoef, J.A. Hermesen, D.A. Van Dartel, J.L. Pennings, and A.H. Piersma, Triazole-induced concentration-related gene signatures in rat whole embryo culture. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): Platform Abstract 5.
145. Bahn, S. and B.F. Hales, Maternal exposure of hydroxyurea activates DNA damage response signalling pathways in organogenesis stage mouse embryos. *Birth Defects Res A Clin Mol Teratol*, 2012. 94(5): Platform Abstract 8.
146. Goldman, J.M., S.C. Laws, S.K. Balchak, R.L. Cooper, and R.J. Kavlock, Endocrine-disrupting chemicals: Prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Recommendations. *Crit Rev Toxicol*, 2000. 30(2): 135–196.
147. Stoker, T.E., L.G. Parks, L.E. Gray, and R.L. Cooper, Endocrine disrupting chemicals: Prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommendations. *Crit Rev Toxicol*, 2000. 30(2): 197–252.
148. Blair, R.M., H. Fang, W.S. Branham, B.S. Hass, S.L. Dial, C.L. Moland, W. Tong, L. Shi, R. Perkins, and D.M. Sheehan, The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol Sci*, 2000. 154(1): 138–153.

**APPENDIX: ADDITIONAL RELATED
INFORMATION (FIGURE 12.A.1;
TABLES 12.A.1 THROUGH 12.A.3)**

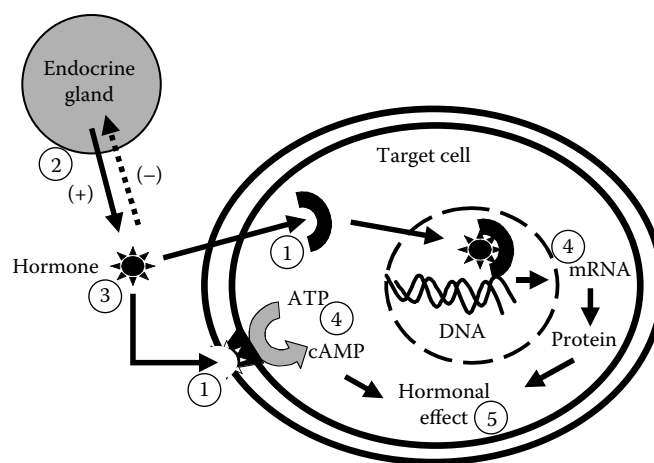


FIGURE 12.A.1 A generalized schematic of mammalian hormonal regulation showing major points for potential disruption/modulation. Endocrine disruptors/modulators are defined as exogenous substances that can alter or modulate endocrine function resulting in adverse effects at the level of the organism, its progeny, and/or populations of organisms. The site of action most focused on for these substances is at the target cell hormone receptor (1). A substance that has an affinity for binding to either a peptide-hormone membrane receptor or a steroid-hormone cytoplasmic receptor might act either as an agonist inducing the hormonal action or as an antagonist, preventing the natural hormone from inducing its effect. However, endocrine disruptors/modulators need not interact with a receptor to affect hormonal regulation. They may affect the synthesis and secretion of the hormone or its regulatory control at the endocrine gland (2) or the transport or elimination of the hormone (3) resulting in increased or decreased levels of hormone reaching the target cell. Endocrine disruptors/modulators might interfere with or alter the cellular mechanisms through which the hormone exerts its effect (4). For steroid hormones, this involves gene activation and synthesis of specific proteins or for peptide hormones, activation of a second-messenger sequence producing cellular effects such as enzyme activation and alteration of cell membrane permeability. Endocrine disruptors/modulators may alter the temporal expression of the hormonal effect (5) such as causing the premature expression of the hormonal effect at a critically sensitive period during sexual development.

TABLE 12.A.1
Reported Female Pubertal Parameters in Various Strains of Rats^{a,b}

Strain	Control VO (Day)	Age—1st E	Age—1st Di	BW at VO
Holtzman	36.8 ± 0.2	NR	NR	NR
Holtzman	35.6 ± 0.7	NR	36.6 ± 0.7	129.7 ± 2.6
Holtzman	35.6 ± 0.8	NR	36.7 ± 0.8	114.9 ± 4.1
Holtzman	34.4 ± 1.2 ^c	NR	NR	NR
	35.0 ± 0.3			
	35.6 ± 1.2			
Sprague–Dawley	35.5 ± 1.0	35.9 ± 0.9	NR	115.9 ± 5.5
Sprague–Dawley	38.4 ± 0.4	NR	NR	180 ± 8 ^{d,e}
Sprague–Dawley*	35.2 ± 0.3	NR	36.6 ± 0.2	90 ± 2 ^e
Sprague–Dawley	34.0 ± 0.2	NR	35.0 ± 0.4	124.3 ± 4.4 ^d
Sprague–Dawley*	31.7 ± 0.4	NR	33.0 ± 0.4	NR
Sprague–Dawley	33.6 ± 0.5	NR	NR	125.9 ± 1.2
Sprague–Dawley	31.9 ± 0.4 ^c	NR	NR	126.1 ± 2.1 ^f
	36.1 ± 0.4			
	37.9 ± 0.5			
	38.3 ± 0.5			
Sprague–Dawley	34.2 ± 0.7 ^g through 37.2 ± 1.5	NR	NR	129.1 ± 3.0 ^h
Sprague–Dawley	33.4 ± 0.8 ⁱ	NR	NR	NR
Wistar	41.7 ± 0.5	NR	NR	NR
Wistar	37.4 ± 3.1	NR	NR	104.8 ± 20.5
Wistar	37.0 ± 0.8	NR	NR	123 ± 2.4
Wistar	36.7 ± 0.3	NR	NR	NR
Wistar	34.3 ± 0.8	35.1 ± 0.8	NR	101 ± 3
Wistar	35.4 ± 1.0	NR	NR	NR
Wistar (R-Amsterdam substrain)	39.3 ± 0.5 ^c	39.5 ± 0.4	NR	90.8 ± 2.0
	38.2 ± 0.8	38.5 ± 0.9		90.6 ± 2.0
Wistar (R-Amsterdam substrain)	38.6 ± 0.4 ^g through 42.1 ± 0.3	NR	NR	93.9 ± 2.8 ^{g,j} through 105.9 ± 1.8
Fischer 344	36.5 ± 1.5	NR	NR	86.6 ± 3.2
Long–Evans	39.0 ± 3.0	NR	NR	NR
Long–Evans	32–34 ^c	33–34 ^c	NR	97–116 ^c
Long–Evans	35.0 ± 0.3	NR	NR	130 ± 2
Long–Evans	36.2 ± 0.5	NR	NR	NR
Long–Evans	30.6 ± 0.2	NR	NR	114 ± 1.8

Source: From Goldman, J.M. et al., *Crit. Rev. Toxicol.*, 30, 135, 2000. With permission.

Notes: *, Data are taken from selected papers that are representative for each of the listed rat strains. VO, vaginal opening; E, vaginal estrus; Di, vaginal diestrus; NR, not reported.

^a Values are group means ± standard errors of the mean.

^b All cited studies employed light:dark photoperiods of 12 h:12 h or 14 h:10 h with the exception of two studies (*) which reported 10 h:14 h.

^c Numbers represent the range of control group means for different experimental blocks.

^d Body weight (BW) at first Di.

^e Estimate from graphed data.

^f BW at Day 35.

^g Group range for a variety of control groups matched against different treatment conditions.

^h BW at Day 37.

ⁱ Mean age ± standard deviation for control rats from studies conducted at Merck Pharmaceutical between 1989 and 1997.

^j BW at first ovulation.

TABLE 12.A.2
Ontogeny of Receptors and Hormones in the Male Rat

Steroid or Protein	GD 18 to Birth	PND 1–15	PND 16–25	PND 26–35	PND 36–45	PND 46–55	PND 56–65	PND 66–90
GnRH pulsatility	+	+	+++	+++	+++	+++	Pulsatile	Pulsatile
GnRH receptor	GD 16–birth +	+	+	+++	+++	+++	++	–
LH receptor	GD 15 +	+	++	++	+++	+++	+++	+++
FSH	+	+	+	+++	+++	++	++	++
FSH receptor semitubules	+	+++ PND 10–15	+++	++	–			
Prolactin	+	+	+	+++	+++	–		
Inhibin B		+ PND 3, ++ PND 10–15	+++	++	+			
TSH (plasma)	+++	+ to +++	++	+++	+++	++	++	++
T3		+ to ++	+++	+++	++	++	+	
T4		+ to ++	+++	+++	+++	++	++	
AR testes	+	++	+	++	++	+++	+++	+++

Source: From Stoker, T.E. et al., *Crit. Rev. Toxicol.*, 30, 197, 2000. With permission.

Notes: –, Decreased to adult levels; +, low level, ++, moderate level, and +++, high level. Blank cells represent undetermined levels. AR, androgen receptor; GD, gestation day; GnRH, gonadotropin-releasing hormone; PND, postnatal day.

TABLE 12.A.3

Estrogen Receptor IC₅₀ Values and Relative Binding Affinities for Various Substances

Substance	Mean IC ₅₀ (M)	Relative Binding Affinity (%)	Log Relative Binding Affinity
17β-Estradiol	8.99 × 10 ⁻¹⁰	100.0	2.00
17α-Estradiol ^a	2.93 × 10 ⁻⁸	3.068	0.49
Estriol	9.25 × 10 ⁻⁹	9.719	0.99
Estrone	1.23 × 10 ⁻⁸	7.309	0.86
Alachlor	>1 × 10 ⁻⁴	—	—
Aldosterone	>1 × 10 ⁻⁴	—	—
Aldrin	>6 × 10 ⁻⁴	—	—
Benzyl alcohol	>1 × 10 ⁻²	—	—
Benzylbutyl phthalate	>1 × 10 ⁻³	—	—
Bis(2-ethylhexyl)phthalate	>1 × 10 ⁻³	—	—
2,2-Bis(4-hydroxyphenyl)butane (bisphenol B)	1.05 × 10 ⁻⁶	0.086	-1.07
Bisphenol A	1.17 × 10 ⁻⁵	0.008	-2.11
Caffeine	>1 × 10 ⁻⁴	—	—
Corticosterone	>1 × 10 ⁻⁴	—	—
<i>o,p'</i> -DDD	>3 × 10 ⁻⁴	—	—
<i>o,p'</i> -DDT	6.43 × 10 ⁻⁵	0.001	-2.85
<i>p,p'</i> -DDT	>1 × 10 ⁻³	—	—
Dibutyl phthalate	>1 × 10 ⁻³	—	—
2,4'-Dichlorobiphenyl	3.65 × 10 ⁻⁴	0.0002	-3.61
2,4-Dichlorophenoxyacetic acid (2,4-D)	>1 × 10 ⁻⁴	—	—
Dieldrin	>1 × 10 ⁻⁴	—	—
Diethyl phthalate	>1 × 10 ⁻³	—	—
Diethylstilbestrol	2.25 × 10 ⁻¹⁰	399.556	2.60
5α-Dihydrotestosterone ^a	>1 × 10 ⁻³	—	—
Dihydroxymethoxychlor olefin	3.40 × 10 ⁻⁸	2.644	0.42
2,2'-Dihydroxybenzophenone	>1 × 10 ⁻⁴	—	—
4,4'-Dihydroxybenzophenone	2.60 × 10 ⁻⁵	0.003	-2.46
4,4'-Dihydroxystilbene ^a	3.20 × 10 ⁻⁷	0.281	-0.55
Dopamine	>1 × 10 ⁻⁴	—	—
Ethinyl estradiol ^b	4.73 × 10 ⁻¹⁰	190.063	2.28
2-Ethylhexyl-4-hydroxybenzoate ^a	4.95 × 10 ⁻⁶	0.018	-1.74
4-Ethylphenol	1.34 × 10 ⁻³	0.00007	-4.17
Eugenol	>1 × 10 ⁻³	—	—
Hepatochlor	>1 × 10 ⁻⁴	—	—
2-Hydroxy-4-methoxybenzophenone	>1 × 10 ⁻⁴	—	—
4-Hydroxytamoxifen	5.13 × 10 ⁻¹⁰	175.244	2.24
Kepone	7.00 × 10 ⁻⁶	0.013	-1.89
Lindane	>1 × 10 ⁻⁴	—	—
Melatonin	>1 × 10 ⁻⁴	—	—
Methoxychlor	1.44 × 10 ⁻⁴	0.001	-3.20
Metolachlor	>1 × 10 ⁻⁴	—	—
Mirex	>1 × 10 ⁻⁴	—	—
Nafoxidine	1.25 × 10 ⁻⁷	0.719	-0.14
4-Nonylphenol ^a	2.40 × 10 ⁻⁶	0.037	-1.43
1,8-Octanediol	>1 × 10 ⁻⁴	—	—
4-Octylphenol	1.95 × 10 ⁻⁵	0.005	-2.34
4-Phenethylphenol	4.40 × 10 ⁻⁵	0.002	-2.69
Phenolphthalin	4.25 × 10 ⁻⁴	0.0002	-3.67
2-Phenylphenol	>1 × 10 ⁻⁴	—	—
3-Phenylphenol	2.45 × 10 ⁻⁴	0.0004	-3.44
4-Phenylphenol	9.80 × 10 ⁻⁵	0.001	-3.04

(continued)

TABLE 12.A.3 (continued)
Estrogen Receptor IC₅₀ Values and Relative Binding Affinities for Various Substances

Substance	Mean IC ₅₀ (M)	Relative Binding Affinity (%)	Log Relative Binding Affinity
Progesterone	$>1 \times 10^{-3}$	—	—
Propyl 4-hydroxybenzoate	1.50×10^{-4}	0.0006	-3.22
4-Stilbenol	$>1 \times 10^{-4}$	—	—
Tamoxifen citrate	5.55×10^{-8}	1.620	0.21
4- <i>tert</i> -Amylphenol	1.65×10^{-4}	0.0005	-3.26
4- <i>tert</i> -Butylphenol	3.68×10^{-4}	0.00024	-3.61
Testosterone	$>1 \times 10^{-3}$	—	—
2',3',4',5'-tetrachloro-4-biphenylol ^a	3.95×10^{-7}	0.228	-0.64
Thalidomide	$>1 \times 10^{-3}$	—	—
Triphenyl phosphate	$>1 \times 10^{-4}$	—	—

Source: Adapted from Blair, R.M. et al., *Toxicol. Sci.*, 54, 138, 2000.

Notes: Data in this table were derived from a standardized estrogen receptor competitive-binding assay. Uteri from ovariectomized Sprague-Dawley rats were the source of the estrogen receptor. The IC₅₀ = substance molar concentration giving 50% inhibition of [³H]-estradiol binding. The relative binding affinity was calculated by dividing the IC₅₀ of estradiol by the IC₅₀ of the substance and is expressed as a percent (estradiol = 100). Refer to the reference paper by Blair et al. for details on the method used and receptor relative binding affinities for additional substances.

^a Substance exhibited a U-shaped binding curve.

^b Synthetic estrogen.

13 Genetic Toxicology

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INTRODUCTION

This chapter on genetic toxicology is designed to serve as a reference guide to toxicologists and regulatory specialists developing genotoxicity data for product evaluation and regulatory submission. Human exposure to genotoxic chemicals is of regulatory concern due to the association of DNA damage in somatic cells with cancer and other diseases, and DNA damage to germ cells leading to heritable mutations in future generations. Assays for measuring genotoxicity have been in use for over 40 years. There are hundreds of genotoxicity assays and they are generally grouped by the end point that is measured, such as assays for mutations, chromosome alterations, primary DNA damage, and DNA repair. These end points can be measured in bacteria, yeast, fungi, plants, invertebrates, mammalian cells in culture, animals, and humans. DNA damage potential can be assessed at all stages of drug or chemical development including the use of computer modeling, simple screening assays for lead optimization, standard GLP testing for regulatory submission, and more complex GLP studies to investigate specific issues to help assess human risk. Out of hundreds of assays, a standard battery of tests used within industry and regulatory agencies has been defined with some variations depending on whether the testing is for drugs, cosmetic ingredients, chemicals, etc. Briefly, the standard battery consists of both *in vitro* and *in vivo* genotoxicity assays. *In vitro* assays are used for an initial assessment since they have the advantage of being relatively inexpensive and quick. *In vivo* assays provide a more biologically relevant assessment of genotoxicity since they reflect normal distribution and metabolism, but require the use of animals and are more time-consuming and costly than *in vitro* assays.

The individual sections of this chapter are designed to provide a general understanding of the principles of each test and a consideration of factors that must be weighed in assay performance and data evaluation. The role of *in silico* computer modeling of DNA-damaging potential and predictive *in vitro* screening assays is discussed. Reviews are provided for the most commonly performed genetic toxicology tests, including bacterial mutation assays, *in vitro* mammalian mutation assays, *in vitro* and *in vivo* assays for chromosome alterations, and unscheduled DNA synthesis. In addition, reviews of some newer assays or existing assays that have had renewed interest are included, such as comet, Pig-a, *in vivo* transgenic mutation, and *in vitro* cell transformation assays. Finally, a summary is provided of regulatory requirements for agencies in the United States, the European Community, and Japan. It is important that prior to conducting testing for regulatory submission the guidelines for the specific agencies are reviewed as testing requirements are periodically updated.

IN SILICO TOXICOLOGY

Computer (*in silico*) modeling permits predictions of biological activity to be made based on chemical structure without the need for actual laboratory testing. *In silico* toxicology

software programs use various approaches to correlate existing toxicology data with chemical structure to predict the toxicity of a chemical. Such programs that correlate chemical structure with biological activity are called Structural Activity Relationship (SAR) programs. These programs can be further subdivided based on the approach used for data analysis. Software based on machine learning, statistical analysis, or molecular topology modeling fall into the (Quantitative) Structural Activity Relationship or (Q)SAR programs. Other approaches based on human knowledge are called Expert Systems.

While SAR analysis is not a substitute for actual *in vitro* or *in vivo* testing, it can provide insight into potential genotoxic hazard of a molecule. Computational toxicology models can provide valuable information during the discovery and lead optimization process by identifying potential adverse genotoxic effects in candidate chemicals—whether a drug, pesticide, impurity, degradant, or metabolite. SAR analysis can then help guide modification of problematic structures to reduce potential adverse effects.

SAR analysis has come into routine usage by various regulatory agencies. It is a central focus of the Critical Path Initiative¹ toolkit for predicting toxicities early in drug development at the US Food and Drug Administration, Center for Drug Evaluation and Research (CDER). An example of the use of SAR by the FDA is in the draft guidance to industry for dealing with genotoxic and carcinogenic impurities in drugs.² This approach for qualifying drug impurities is expected to be included in future guidance to the pharmaceutical industry for the qualification of impurities in drugs through the International Conference on Harmonisation (ICH) process.³ It is reasonable to assume that a person involved in drug, chemical, or other product development or regulation will use SAR data in some way in the future. Using a slightly different approach, the US Environmental Protection Agency, National Center for Computational Toxicology (EPA NCCT) has developed an *in silico* modeling program called ToxCast™ to aggregate high-throughput screening data, including genetic toxicology data, to prioritize chemicals based on hazard in different pathways of toxicity.⁴ In Europe, the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) program has made QSAR a central part of their product safety program by developing a “toolbox” for the *in silico* evaluation of industrial chemicals for hazard.^{5,6}

There are biochemical and biophysical properties of chemicals that correlate with chemical-induced genotoxicity. The relationship of chemical structure, genotoxicity, and carcinogenicity was elegantly described by Ashby and Tennant forming the concept of “structural alerts” for genotoxic carcinogens.⁷ The ability of a chemical to consistently interact with DNA through specific mechanisms is fundamental to the process of genotoxicity, and in turn, to the prediction of genotoxicity based on chemical structure. For example, a chemical with an electrophilic functional group and the ability to form a covalent bond with DNA is the prototypic structure associated with DNA damage leading to a structural alert. Another classic mode of

action is intercalation of a chemical into the DNA double helix by planar molecules, such as polycyclic aromatic hydrocarbons. At the most basic level, the DNA-damaging molecule must reach the DNA to exert its effect. For this reason, other properties such as reactivity, stability, metabolism, aqueous solubility, and the ability to cross lipid membranes are key attributes for a chemical to gain access to bacterial or eukaryotic DNA. The original empirical expert observations have matured into sophisticated *in silico* models using disciplines such as molecular topology and mathematical chemistry to predict biological activity based on chemical structure.

There are some limitations to using *in silico* modeling to identify genotoxic agents and in turn genotoxic carcinogens. Novel chemicals without thorough representation in the training data sets may give inaccurate predictions. Metals, organometallic compounds, fibers, insoluble materials, large molecules (proteins, peptides, and polysaccharides), gases, and complex mixtures cannot be accurately modeled. Many SAR programs focus on identification of genotoxic carcinogens as that type of direct acting carcinogen is of primary concern to regulators. Some expert SAR programs such as DEREK Nexus have better coverage of structural alerts for nongenotoxic carcinogens than other SAR programs.

There are various *in silico* SAR software systems and associated databases available that have evolved in scope, name, and ownership over the years. There is a mixture of databases and software in use, including proprietary systems maintained by individual companies, government databases such as the FDA CDER or EPA NCCT, and commercial databases and software.^{8–10} Some are free and others are commercial products. A discussion of the various SAR programs, approaches, strengths, and weaknesses is beyond the scope of this chapter. Table 13.1 provides information on a number of the more common programs.

TABLE 13.1
Representative *In Silico* SAR Programs for Genetic Toxicology

Program Type	Name	Organization	Website
QSAR	MC4PC	MultiCASE, Inc.	http://multicase.com/
	Nonhuman Genetic Toxicity Suite	Leadscope, Inc.	www.leadscope.com
	SciQSAR	Symyx-MDL, Inc.	www.scimatics.com
Expert systems	Derek-Nexus (w/Meteor)	Lhasa, Ltd.	www.lhasalimited.org
	Oncologic	US EPA	http://www.epa.gov/oppt/sf/pubs/oncologic.htm

BACTERIAL MUTAGENESIS ASSAY

Of the microbial test systems that have been developed over the past 30 years, the *Salmonella typhimurium* and the *Escherichia coli* tester strains are the most widely used for detecting gene mutations. The bacterial reverse mutation assay, commonly known as the Ames test, has proved to be a reliable and economical assay for routine screening of chemicals for mutagenic activity.^{11–15}

The *S. typhimurium* and *E. coli* strains each have a defect in one of the genes involved in histidine or tryptophan biosynthesis, respectively. The defect renders the cell dependent (auxotrophic) on exogenous histidine or tryptophan. Unless the cell experiences a mutation that reverts the dysfunctional gene back to the wild-type (prototrophic) genotype, the cell ceases to grow when the exogenous histidine or tryptophan is exhausted. For this reason, this assay is referred to as a reverse mutation assay. Since base substitution mutations are reverted only by base substitution mutagens and frameshift mutations are reverted only by frameshift mutagens, it is necessary to use more than one tester strain to detect both types of mutagens. *S. typhimurium* strains, identified with a prefix “TA,” detect reversion from his[−] to his⁺ at a single site in one of the 12 steps of histidine biosynthesis. *E. coli* strains, identified with a prefix WP2, detect reversion from trp[−] to trp⁺ at the *trpE* gene in a site blocking tryptophan biosynthesis prior to the formation of anthranilic acid. To adequately assess the mutagenic potential of a chemical, it is important to select an appropriate battery of tester strains. The strain battery that was globally adopted in 1997 by the Organisation for Economic Cooperation and Development (OECD)¹⁶ and the ICH¹⁷ requires selection of one strain from each of five categories as follows:

1. TA98
2. TA100
3. TA1535
4. TA1537, TA97, or TA97a
5. TA102, WP2 *uvrA*, or WP2 *uvrA* (pKM101)

To detect cross-linking agents, strain TA102 or WP2 (pKM101) must be used.

Strains TA98, TA1537, TA97, and TA97a are reverted by frameshift mutagens. Strains TA100, TA1535, TA102, WP2 *uvrA*, and WP2 *uvrA* (pKM101) are reverted by base substitution mutagens.^{18,19} Strain TA102 and the *E. coli* strains possess A–T base pairs at the site of the mutation, while the other strains possess G–C base pairs at their mutation sites.

To increase their sensitivity to mutagens, additional mutations have been incorporated in each strain. Mutations in the *uvrA* gene of *E. coli* and in the *uvrB* gene of *S. typhimurium* are partial deletions of each respective gene.²⁰ The *uvr* genes code for a series of DNA excision repair proteins involved in removal of T–T dimers induced by ultraviolet (UV) light. Cells with this type of mutation are unable to repair damage induced by UV light and other types of mutagens. The presence of either of these mutations can be detected by

demonstrating sensitivity to UV light. The pKM101 plasmid codes for an error-prone DNA repair system.^{21,22} The proposed mechanism by which this plasmid increases sensitivity to mutagens is by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.^{23–25} Cells containing this plasmid are resistant to ampicillin. The *rfa* wall mutation prevents the *S. typhimurium* cells from synthesizing an intact polysaccharide cell wall.²⁶ Therefore, large molecules such as benzo[a]pyrene (BaP) that are normally excluded are able to penetrate the cell. Cells containing this mutation are sensitive to crystal violet. In addition, TA102 has been shown to be useful for detecting oxidative mutagens such as bleomycin that are not detected by other tester strains. The *uvrB* mutation has not been introduced into this strain; therefore, with an intact excision repair system, it can detect cross-linking agents such as mitomycin C (MMC).²⁷

Although the tester strains described earlier are capable of detecting a wide range of direct-acting mutagens, they are incapable of detecting promutagens until the promutagens are converted to their mutagenic forms by NADPH-dependent mammalian microsomal enzymes. Since bacteria do not possess the microsomal enzymes necessary for promutagen activation, the required metabolic activity must be supplied exogenously. Ames et al.²⁸ developed an exogenous metabolic activation system derived from the microsomal fraction of mammalian tissue homogenates. The supernatant (referred to as S9) from centrifugation of the liver homogenate at 9000 g is combined with necessary cofactors for use as an S9 mix in the bacterial mutagenesis assay.

Bacterial mutation assays can be performed using either the more common plate incorporation procedure or the preincubation modification of the assay. In the plate incorporation procedure, a 100 μ L aliquot of tester strain, 50–1000 μ L of test or control article, and 500 μ L of S9 mix or buffer are added to 2.0 mL of molten selective top agar. After mixing, the mixture is overlaid onto the surface of a Vogel–Bonner bottom agar plate. After the plates have solidified, the plates are inverted and incubated for ~ 48 h at $37^\circ\text{C} \pm 2^\circ\text{C}$ prior to counting revertant colonies.

The standard plate incorporation assay using Aroclor 1254-induced rat liver S9 is not effective for detecting all classes of promutagens. The preincubation modification described by Yahagi et al.²⁹ has greatly enhanced the utility of the bacterial mutation assay. A number of chemicals are more readily detected using the preincubation approach. These include various nitrosamines,²⁹ certain azo compounds,³⁰ and classical mutagens such as aflatoxin B₁, ben-zidine, and BaP.³¹ The preincubation methodology allows for maximum interaction between tester strain, S9, and test chemical, which could explain its increased sensitivity to certain chemicals, especially for volatile chemicals and for test materials that are labile in aqueous systems.

In the preincubation assay, the tester strains are incubated with the test article in a liquid environment prior to plating. A 50–1000 μ L aliquot of test or control article,

500 μ L of S9 mix or buffer, and 100 μ L of tester strain are added to glass culture tubes. The mixture is allowed to incubate for the appropriate period of time (e.g., 20 or 60 min at $37^\circ\text{C} \pm 2^\circ\text{C}$). Selective top agar (2.0 mL) is then added to each tube and the mixture is overlaid onto the surface of a Vogel–Bonner minimal bottom agar plate. After the plates have solidified, the plates are inverted and incubated for ~ 48 h at $37^\circ\text{C} \pm 2^\circ\text{C}$ prior to counting revertant colonies.

The following criteria must be met for the mutagenicity assay to be considered valid:

1. *Tester strain integrity.* The presence of the *rfa* wall mutation must be confirmed in the *Salmonella* strains by demonstrating sensitivity to crystal violet. The presence of the *uvrA* or *uvrB* mutation must be confirmed by demonstrating sensitivity to UV light. The presence of the pKM101 plasmid must be confirmed by demonstrating resistance to ampicillin. The presence of the pAQ1 plasmid must be confirmed by demonstrating resistance to tetracycline.
2. *Spontaneous revertant background frequency.* All tester strains must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. Each laboratory must define its own acceptable frequency based on historical control data and published ranges. The consistency of the solvent control values among the more commonly used solvents (water, dimethyl sulfoxide [DMSO], ethanol, and acetone), using both the plate incorporation and preincubation methods, has been demonstrated.³²
3. *Tester strain titers.* To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be $\sim 10^9$ cells/mL.
4. *Positive control values.* Each mean positive control value must exhibit at least a twofold increase over the respective mean vehicle control value for tester strains with the exception that a threefold increase is needed for TA1535, TA1537, and WP2 *uvrA*. Two positive control chemicals are routinely included for each tester strain in each assay: one that is directly acting and positive in the absence of an exogenous source of metabolic activation and one that is indirectly acting and requires metabolic activation to its active form. A commonly used positive control chemical in the presence of exogenous metabolic activation is 2-aminoanthracene for *Salmonella* tester strains TA98, TA100, TA1535, TA1537, TA97, and TA97a and for *E. coli* tester strains WP2 *uvrA* and WP2 *uvrA* (pKM101). Sterigmatocystin is a positive control used for tester strains TA102 and WP2 (pKM101) with metabolic activation. For positive control chemicals in the absence of exogenous metabolic activation, the following are

commonly used: sodium azide for TA100; and TA1535, 2-nitrofluorene for TA98; 9-aminoacridine for TA1537, TA97, and TA97a; and methyl methanesulfonate (MMS) for all *E. coli* tester strains. MMC is commonly used for tester strain TA102 without activation.

5. **Toxicity.** A minimum of three nontoxic dose levels is required for an acceptable assay. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean solvent control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that fewer than three nontoxic dose levels are achieved, the affected portion of the assay is repeated with an appropriate change in dose levels.

All conclusions are based on sound scientific judgment; however, as a guide to interpretation of the data, the following criteria may be considered. Although there is no evidence supporting a specific requirement for a two- or threefold increase over background, this rule of thumb is the most common method used in the evaluation of data. For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Data sets for tester strains TA98, TA100, TA102, TA97, TA97a, and *E. coli* WP2 *uvrA* (pKM101) are judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean solvent control value. Data sets for tester strains TA1535, TA1537, and WP2 *uvrA* are judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean solvent control value. Ideally, the most appropriate determinant of a positive (mutagenic) response is a reproducible, statistically significant dose-related increase in revertant colonies. Several statistical procedures have been published; however, a recommended choice has not been globally adopted.^{33,34}

IN VITRO MAMMALIAN CELL GENE MUTATION ASSAYS

The most common *in vitro* assays for gene mutation in mammalian cells utilize cultured mouse lymphoma L5178Y cells or Chinese hamster ovary (CHO) cells. Both assays are designed to detect forward mutations at specific loci and are designated as the thymidine kinase^{+/−} (TK^{+/−}) mouse lymphoma mutation assay³⁵ and the CHO/hypoxanthine–guanine phosphoribosyl transferase (HPRT or HGPRT) mutation assay,³⁶ according to their respective target genes, *tk* and *hprt*. The two assays, described by OECD Test Guideline 476,³⁷ differ procedurally in certain areas, such as the way cells are cultured and cloned and the time for the expression

and selection periods, but follow the same basic principles. They have the following steps in common:

1. Preparation of cells for treatment
2. A preliminary cytotoxicity test
3. Treatment with the test article in the presence and absence of an exogenous metabolic activation system (S9)
4. An expression period during which mutations are fixed in the DNA and endogenous levels of wild-type enzyme decrease
5. A selection period during which mutant cells are cloned in the presence of a selective agent
6. Cytotoxicity and cloning efficiency determinations
7. Data collection, calculations to determine mutant frequency, cytotoxicity and cloning efficiency, and an assessment of the test results

L5178Y TK^{+/−} MOUSE LYMPHOMA MUTATION ASSAY

The TK^{+/−} mouse lymphoma mutation assay (MLA) utilizes a strain (of mouse lymphoma cells TK^{+/−}–3.7.2C clonal line) that is heterozygous at the TK locus. These cells contain functional TK enzyme, which is involved in a salvage pathway for use/reuse of thymidine in the cell through phosphorylation of thymidine. Trifluorothymidine (TFT), the selective agent used in this assay, can also be phosphorylated by the TK enzyme, but is lethal to the cell. Forward mutation at the single functional TK gene forces the cell to switch to *de novo* thymidine synthesis. The loss of TK activity in mutant cells (the cells are tk^{−/−}) makes the mutant cells resistant to TFT (TFT^r) and able to grow in the presence of TFT.

L5178Y cells are initially cultured, treated 4 h with and without S9 or 24 h without S9, and cultured in suspension for 2 days to permit the phenotypic expression of induced mutants. Treated cells are then plated for mutant selection with the selective agent TFT and a lower density without TFT to determine plating efficiency. Mutant colonies that arise in soft agar are counted with an electronic colony counter after 10–14 days of incubation in a soft agar medium containing TFT.

The MLA and CHO/HPRT assays both detect point mutations involving base substitutions, deletions, frame shifts, and rearrangements within the locus and can be used interchangeably for detection of these types of lesion. However, the MLA also detects clastogenic lesions involving multiple genes and multilocus deletions.³⁸ These different types of mutations, point mutations vs. multilocus deletions, can be distinguished by TFT^r mutant colony size. After treatment with many mutagenic substances, TFT^r colonies exhibit a characteristic bimodal size distribution, which can be determined through the use of an electronic colony counter with sizing capability. The distribution of large and small mutant colonies is characteristic of the types of mutations induced.^{38,39} There is cytogenetic and molecular evidence indicating small colony variants carry chromosome aberrations or rearrangements involving with

chromosome 11, on which the TK locus is located in the mouse.^{40,41} In contrast, large mutant colonies are karyotypically similar to the parental cells and contain presumptive point or intralocus mutations. Thus, the MLA makes it possible to detect a variety of genetic lesions, and to discriminate between point mutations and multilocus mutations.

Maintenance of an acceptable background mutant frequency is essential. L5178Y cells are routinely cleansed of spontaneous mutant cells by supplementing medium with thymidine, hypoxanthine, methotrexate, and glycine. An acceptable background mutant frequency can be established by evaluating untreated and/or vehicle controls. Concurrent untreated controls are not required for an assay, but inclusion would be recommended when utilizing a nonstandard vehicle. Historical vehicle control mutant frequency is reported as mutants per 10^6 surviving cells with range and standard deviation. Normally, data are continuously updated, with the most recent 3 years included in a report.

Two positive controls, one direct acting and one requiring S9, are also included with each assay. MMS, a direct-acting mutagen that induces both large and small colonies, and dimethylbenz(a)anthracene, which requires S9, are used. In addition, MMS serves as a control for the ability to detect small mutant colonies.⁴² Historical positive control data are also included in a report as described earlier.

The cytotoxic effects of the treatment must be determined to decide the doses evaluated in the definitive assay. Doses that produce >90% reduction in survival as compared with the negative controls are excluded from evaluation as excessively cytotoxic. Currently, 80%–90% reduction in survival is considered optimal for detecting mutagenic activity of a cytotoxic test agent. For test compounds that are not cytotoxic or insoluble, demonstration by chemical analysis that appropriate doses were used becomes more critical.

Cytotoxicity or survival is expressed as % Relative Total Growth (RTG), which is determined by multiplying the % Suspension Growth from the expression period by the % Cloning Efficiency at the time of selection. The % Suspension Growth is determined by comparing the daily growth of treated cells vs. negative control cells during the 2 day phenotypic expression period. The % Cloning Efficiency is determined by comparing the number of colonies that form under nonselective conditions with the number of cells plated.

Certain other considerations are important to the validity of the assay. In general, test compounds are tested up to a concentration of 5 mg/mL or 10 mM (500 mg/mL or 1 mM for pharmaceuticals¹⁷), up to or in slight excess of the limits of solubility, or up to doses that produce 80%–90% RTG. Doses that produce little or no toxicity should also be included. In addition, control of pH and osmolality is critical since excesses of either may induce irrelevant positive results. New lots of S9, serum, and medium should be screened for their ability to support growth and to keep background mutant frequencies within acceptable levels. Acceptable cloning conditions are best judged by the recovery of small colony mutants as determined by sizing of positive control colonies.

The validity of the assay is determined by the following criteria:

- The average spontaneous mutant frequency of the vehicle control cultures must be within 35–140 TFT-resistant mutants per 10^6 surviving cells. Low spontaneous mutant frequencies, that is, 20–34 mutants per 10^6 surviving cells, are considered acceptable if small colony recovery is demonstrated.⁴³ The average cloning efficiency of the vehicle controls must be between 65% and 120% and the total suspension growth between 8% and 32% for the 4 h exposure, and 20% and 180% for the 24 h exposure.^{44–46}
- At least one dose of each positive control must meet the criteria for a positive response. In addition, at least one dose of one of the positive controls must produce an induced mutant frequency (IMF) $\geq 300 \times 10^{-6}$ mutants with 40% small colonies, or small colony IMF $\geq 150 \times 10^{-6}$ mutants.^{45,46}

The cytotoxic effects of each treatment are expressed relative to the vehicle control for suspension growth over 2 days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency for each treatment condition is calculated by dividing the mean number of colonies on the TFT plates by the mean number of colonies on the VC plates, multiplying by the dilution factor (2×10^{-4}), and expressed as TFT-resistant mutants per 10^6 surviving cells. The IMF is defined as the mutant frequency of the treated culture minus the mutant frequency of the vehicle control cultures. The International Workshop on Genotoxicity established a Global Evaluation Factor (GEF) for a positive response at an IMF of ≥ 90 mutants per 10^6 clonable cells at the Aberdeen meeting in 2003.⁴⁶

In evaluation of the data, increases in IMF which occur only at highly toxic concentrations (i.e., <10% total growth) are not considered biologically relevant. All conclusions should be based on scientific judgment; however, the following criteria are presented as a guide to interpretation of the data.⁴⁶

- A result is considered positive if a concentration-related increase in mutant frequency is observed in the treated cultures *and* one or more concentrations with $\geq 10\%$ RTG exhibit induced mutant frequencies ≥ 90 mutants per 10^6 clonable cells (based on the average mutant frequency of duplicate cultures). If the average vehicle control mutant frequency is >90 mutants per 10^6 clonable cells, a doubling of mutant frequency over the vehicle is also required.⁴⁴
- A result is considered negative if the treated cultures exhibit induced mutant frequencies of <90 mutants per 10^6 clonable cells (based on the average mutant frequency of duplicate cultures) and there is no concentration-related increase in mutant frequency.

CHO/HPRT MUTATION ASSAY

The CHO/HPRT mutation assay is designed to select for mutations at the X-linked, hemizygous HPRT gene (*hprt*). HPRT catalyzes phosphorylation of purines in the purine salvage pathway. The selective agent used in this assay, 6-thioguanine (6-TG), is also a substrate for this enzyme and cells that retain functional HPRT are killed by 6-TG. Forward mutations that result in the loss of the functional *hprt* render cells resistant to 6-TG (TG^r). These mutant cells can be counted following an expression period by cloning in culture medium supplemented with 6-TG.³⁶

Unlike L5178Y cells, CHO cells are typically treated (for five hours \pm S9), subcultured, and cloned as monolayer cultures. In the authors' laboratory, a 7–9 day mutant expression period is followed by a 7–10 day selection period. After the selection, mutant colonies are fixed, stained, and counted by eye or automated colony counter.

The CHO/HGPRT mutation assay detects point mutations involving base substitutions, frame shifts, deletions, and rearrangements within the *hprt* locus. In order to maintain an acceptable background mutant frequency, CHO cells may be cleansed for removal of preexisting TG^r mutants by supplementing medium with hypoxanthine, aminopterin, and thymidine. Historical vehicle control mutant frequency is reported as mutants/10⁶ clonable cells with range and standard deviation. Normally, data are continuously updated, with the most recent 3 years included in a report.

For the CHO/HPRT mutation assay, ethyl methanesulfonate (EMS), a direct-acting mutagen, and BaP, which requires S9, are routinely used as positive controls.⁴⁷ Historical positive control data are presented as described earlier.

The cytotoxic effects of the treatment must be determined to decide on the concentrations evaluated in the definitive assay. Concentrations that produce 10% survival or less as compared with the negative controls are excluded as excessively cytotoxic. Currently, achievement of 10%–20% relative survival is considered optimal for detecting mutagenic activity from a cytotoxic test agent. It is also appropriate to account for relative cell density after treatment in those cases where there is an appreciable decrease in cell numbers at the end of treatment (adjusted relative survival).³⁶ For test compounds that are not cytotoxic or insoluble, demonstration by chemical analysis that appropriate concentrations were evaluated is critical.

Cytotoxicity is expressed as initial survival (or adjusted relative survival), and is determined by comparing the relative cloning efficiency of treated vs. vehicle control cultures (adjusted for relative cell density after treatment). Survival may be determined immediately after treatment, or following an overnight recovery period.

In general, test compounds are tested up to a concentration of 5 mg/mL (or 10 mM), up to or in slight excess of the limits of solubility, or up to doses that produce 10%–20% survival as compared with the negative controls. Doses that produce little or no toxicity should also be included. In addition, control of pH and osmolality is critical since excesses

of either may induce irrelevant positive results. New lots of S9, serum, and medium must be screened for their ability to support growth and to keep background mutant frequencies within acceptable levels.

The validity of the assay is determined by the following criteria:

- The average absolute cloning efficiency of vehicle controls must be >60% (at initial survival and selection). In addition, the average spontaneous mutant frequency of the vehicle controls must be <20 (and more typically below 10) TG^r mutants/10⁶ clonable cells.
- The positive controls must induce a significant increase in mutant frequency as compared to the concurrent vehicle controls ($p \leq 0.01$).

Statistical methods, such as a transformation test by Snee and Irr,⁴⁸ may be used for interpretation of results of these assays.

The test article is considered to have produced a positive response if it induces a statistically significant, dose-dependent increase in mutant frequency as compared to the concurrent vehicle controls. Typically, a minimum threshold is also included for biological relevance (e.g., ≥ 15 TG^r mutants/10⁶ clonable cells over the concurrent vehicle controls, based upon historical data from the testing laboratory as well as published results). If only one criterion is met (statistically significant OR dose-dependent OR net increase of 15 TG^r mutants/10⁶ clonable cells), the result may be considered equivocal. If none of the criteria are met, the results will be considered to be negative.

ASSAY SYSTEMS FOR CHROMOSOME DAMAGE

Genotoxicity can be evaluated by studying the physical integrity of the chromosomes in toxicant-exposed cultured cells, experimental animals, or even in cells derived from humans exposed *in vivo*. Chromosome preparations can be initiated from any dividing cell population and can even be prepared from nondividing cells, such as peripheral blood lymphocytes (PBL), that can be stimulated by mitogens to divide in culture system for a brief period of time. In contrast to mutagenesis assays that can identify genetic alterations related to specific gene expression or to the identification of specific DNA base mutations by the use of molecular biology techniques, the employment of various chromosome assays allows for direct visualization of the structural integrity of the genome. The state of the genome can be visually observed by routine Giemsa staining of each individual chromosome to look for aberrations in the typical chromosome size, form, and number known for each species. In addition to the direct analysis of chromosomes, an indirect analysis of chromosome damage involving examination of cells for micronuclei can also be performed. Micronuclei originate from chromosome fragments or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase stage of cell division. Together, these different analyses are termed cytogenetic analyses.

IN VITRO CHROMOSOME ABERRATION ASSAY

In vitro chromosome aberration assays are used to measure genotoxicity at the chromosome level. End points measured include changes in chromosome structure (clastogenicity) and chromosome number (aneugenicity). Test systems used include permanent cell lines, such as CHO, Chinese hamster lung (V79 or CHL), human lymphoid cell line (TK6), or primary cells such as human PBL (HPBL) and rat blood lymphocytes. Primary lymphocytes need to be stimulated to divide using a mitogen such as phytohemagglutinin. To mimic the major activity of metabolism and detoxification that may occur *in vivo*, the *in vitro* assays are conducted both in the absence and presence of an exogenous source of metabolic activation. The most commonly used activation system is a mixture of Aroclor 1254–induced rat liver S9 and enzyme cofactors. Following exposure of cells to a test article, dividing cells are arrested in metaphase using a spindle inhibitor (e.g., Colcemid®). Metaphase cells are collected at a time selected to assure that cells are in the first metaphase after chemical exposure, generally 1.5–2.0 times the normal (i.e., untreated) cell cycle length either after the beginning or at the end of treatment. Additional sample times are also selected to allow evaluation of cells whose cell cycle may be delayed and may require more time to reach metaphase. In the event that multiple sample times are not used, the effect of the test article on the normal cell cycle kinetics must be taken into account when selecting the single sampling time.⁴⁹

It is essential that a laboratory conducting such assays should demonstrate its ability to reliably and accurately detect substances of known aneugenic and clastogenic activity, with and without metabolic activation, as well as known negative substances, using the reference substances described in the OECD Test Guideline 473 which is currently being updated.⁵⁰ The laboratory should establish acceptable ranges for the negative control groups. For *in vitro* assay systems, controls often include a negative control consisting of untreated culture as well as a control consisting of solvent alone. For those laboratories with very large historical databases, it may be sufficient to perform only the solvent control for comparison purposes. For small databases or instances requiring the use of an unusual or infrequently used solvent, both the negative and solvent controls are necessary to demonstrate that the solvent did not alter the spontaneous background frequency encountered with the particular target cell line. Aqueous solvents (e.g., water, saline, phosphate buffer) are used at 10% (V/V) while organic solvents (e.g., DMSO, acetone, ethanol, etc.) are used at 1% (V/V) in culture. Results are reported as percent (%) aberrant cells.

Appropriate positive controls are routinely included in each treatment condition of the assay. In general, two positive control chemicals are required: one that is direct acting and positive in the absence an exogenous source of metabolic activation and second that is indirect acting and requires metabolic activation to its active DNA-damaging form. The most commonly used positive controls for the *in vitro* cytogenetics assay systems are EMS or MMC for the

nonactivation test system and cyclophosphamide or BaP for the S9 activation test system. The purpose of these controls is to demonstrate the ability of the target cells to respond to the genotoxic insult. A statistically significant increase in percent aberrant cells in the positive control relative to the solvent control is one criterion for a valid test. Dose levels for positive controls should be selected to yield a moderate level of activity, thereby allowing for evaluation of the sensitivity of the test system.

The cytotoxic effects of treatment must be reported to justify the dose selection for evaluation of chromosome aberrations. Methods most frequently used in this assay include mitotic inhibition (HPBL assay) and cell growth inhibition. A minimum of 50% toxicity must be achieved in the high-dose group for cytotoxic test substances, unless limited by solubility, in which case the lowest precipitating dose level will be the highest dose selected for evaluating chromosome aberrations or other special considerations. The number and types of structural and numerical (polyploidy, endoreduplication) chromosome changes found should be presented for each replicate treatment condition. This information allows consideration of the reproducibility between the replicates as well as the consequence of the damage observed. The percentage of damaged cells (% aberrant cells) in the total population of cells examined is calculated for each group. The frequency of structural aberrations per cell (mean aberrations per cell) is also calculated and reported for each group as a measurement of the severity of damage. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with aberrations or in the frequency of structural aberrations per cell.^{51,52} The percentage of cells with numerical chromosome changes indicates the potential of the test substance to induce aneuploidy.

Statistical analysis of the percent aberrant cells may be performed using a variety of methods; one of the most common is the Fisher's exact test. Fisher's exact test is used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. The Cochran–Armitage test has been recommended as a trend test for dose responsiveness.⁵³

All conclusions are based on sound scientific judgment; however as a guide to interpretation of the data, the test article is considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group.

A number of special considerations, mandated by the properties of the test article, are required with the *in vitro* cytogenetic assays. Materials are normally tested up to or in slight excess of their limit of solubility in the treatment medium. However, because of potentially artifactual results that can be obtained under nonphysiological testing conditions, non-pharmaceuticals are not tested in excess of 10 mM concentrations (or 5 mg/mL)⁵⁴ (this level is being revised in the latest updating of the OECD 473 Test Guideline).⁵⁰ Recently, the ICH has revised their recommendation for genotoxicity testing and data interpretation for pharmaceuticals intended for

human use.¹⁷ As per new ICH recommendation the maximum top concentration recommended is 1 mM or 0.5 mg/mL, whichever is lower, when not limited by solubility in solvent or culture medium or by cytotoxicity. The rationale for a maximum concentration of 1 mM for *in vitro* mammalian cell assays includes the following. The test battery includes the Ames test and an *in vivo* assay. First, this battery optimizes the detection of genotoxic carcinogens without relying on any individual assay alone. There is a very low likelihood of compounds of concern (DNA-damaging carcinogens) that are not detected in Ames test or *in vivo* genotoxicity assay, but are detectable in an *in vitro* mammalian assay only above 1 mM. Second, a limit of 1 mM maintains the element of hazard identification, being higher than clinical exposures to known pharmaceuticals, including those that concentrate in tissues.⁵⁵ Certain drugs are known to require quite high clinical exposures for therapeutic effect, example, nucleoside analogs and some antibiotics. Although comparison of potency with existing drugs can be of interest to sponsors, perhaps even above 1 mM limit, it is ultimately the *in vivo* tests that determine relevance for human safety. For pharmaceuticals with unusually low molecular weight (e.g., <200), higher test concentrations should be considered. The OECD guidelines for the *in vitro* chromosome aberration assay are currently being updated and the maximum concentration may be lowered.

The pH of the treatment medium must also be monitored during the exposure period because of potential artifacts that may be observed under treatment conditions of low or high pH, particularly in the S9-activated test system.⁵⁶ Similarly, ionic strength must be monitored and controlled because of the artifactual responses observed with nonclastogens when tested at concentrations that result in high osmolality.⁵⁷

IN VITRO MICRONUCLEUS ASSAY

The *in vitro* micronucleus (MNvit) assay is used for the detection of micronuclei in the cytoplasm of interphase cells.⁵⁸ Micronuclei originate from acentric chromosome fragments (i.e., lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase stage of cell division. The assay detects the activity of clastogenic (substances that lead to chromosome breakage/damage) and aneugenic (substances that damage the cellular components involved in the segregation of the chromosomes) test chemicals or their metabolites in cells that have undergone cell division after exposure to the test substance.^{59,60}

The MNvit assay can be conducted with or without addition of the cytokinesis blocker, cytochalasin B (cyto B) in cell lines, but the use of cytochalasin B is required for primary HPBL. The use of cytokinesis-blocked method clearly identifies the cells that have undergone nuclear division.⁶¹ Cells grown in the presence of cyto B, an actin polymerization inhibitor, are capable of undergoing a nuclear mitotic division (karyokinesis) but not cytoplasmic division (cytokinesis). Using the cytokinesis-blocked method, a single cell division leads to formation of a binucleated daughter cell

rather than a normal pair of daughter cells (each with one nucleus). When micronuclei are counted in binucleated cells, a true measurement of their induction in dividing cells can be obtained.

When the cytokinesis-blocked method is used, evaluation of cytotoxicity should be based on the Cytokinesis-Block Proliferation Index (CBPI) or Replicative Index (RI). The CBPI indicates the average number of cell cycles per cell during the period of exposure to cytoB, and may be used to calculate cell proliferation.⁵⁹ The RI indicates the relative number of nuclei in treated cultures compared to control cultures and can be used to calculate the % cytostasis. When cytokinesis-blocked method is not used, evaluation of cytotoxicity is recommended based on relative increase in cell counts (RICC) or relative population doubling (RPD), as both take into account the proportion of the cell population which has divided.⁶²

The conduct of the assay is essentially the same as described for the *in vitro* chromosome aberration assay for selection of dose levels and data interpretation. One of the advantages of conducting the MNvit assay is that the origin of micronuclei can be characterized precisely using specialized labeling techniques described later.⁶³

The relative simplicity of the MNvit end point has made it amenable to several automated scoring approaches. These automated methods permit evaluation of larger numbers of cells in relatively less time than manual scoring, therefore, increasing the assay robustness and statistical power. Flow cytometry is one such scoring platform that has been successfully employed.⁶⁴ While the current state-of-the-art methods acquire micronucleus (MN) frequency data very efficiently, it is becoming clear that they also endow the assay with high information content. For instance, simultaneous with MN frequency determinations, several additional end points are acquired that provide insights into cytotoxicity, cell cycle perturbation and, in the event of MN induction, information about genotoxic mode of action.

The flow cytometric technique utilizes a dual dye (ethidium monoazide bromide and SYTOX® Green) sequential staining procedure, at the time of harvest. Intact cells are exposed to ethidium monoazide bromide. This DNA-specific dye stains only the DNA of necrotic and late-stage apoptotic cells. This first dye is washed out, cells lysed, and the resulting nuclei and micronuclei stained with SYTOX® Green. DNA from dead or dying cells fluoresce at two wavelengths from the two dyes and thus data from excessively toxic cells can be excluded from scoring. Nuclei from healthy cells can be identified based on fluorescence at a single wavelength and are included in scoring. Three groups of data are collected from each culture: nuclei, hypodiploid nuclei (nuclei missing one or more chromosomes), and micronuclei (DNA fragments one tenth to one hundredth the mean nuclear diameter). Increase in the ratio of free micronuclei relative to nuclei in treated cultures relative to control cultures is the basis for micronuclei analysis. Similar analysis of the hypodiploid nuclei fraction in treated vs. control cultures permits insight into the genotoxic mode of action for those treatments

resulting in an increase in micronuclei. Clastogens result in an increase in micronuclei but not hypodiploid nuclei while aneugens increase both micronuclei and hypodiploid nuclei. This aneugenic and clastogenic “signature” provides useful information into genotoxic mode of action. Clastogens are of greater concern to regulators than aneugens because clastogens usually cause direct damage to DNA while aneugens operate via indirect mechanisms with thresholds. For this reason, flow cytometric MNvit data are useful in that both the end point is measured and when positive, a mode of action can often be identified.

This technique is suitable with various cell lines (TK6, CHO, and V79). The *in vitro* flow cytometric scoring assay is performed in absence of cytochalasin B, and thus is physiologically more relevant. Primary cells such as PBL are not suitable at present using this technique. However, the use of flow cytometry for scoring for MNvit assays has come into routine use for non-GLP screening assays and with the publication of OECD 487 guideline, is now available for use in GLP studies.⁶⁵

IN VIVO CHROMOSOME ABERRATION ASSAY

The two most frequently used end points for *in vivo* evaluation of clastogenic potential are the bone marrow chromosome aberration assay and the bone marrow MN test. Both assays may be conducted using either mice or rats. To maximize delivery of the test article to the bone marrow and an expected route of human exposure, the route of animal dosing should be justified. The limit for acute dosing for this assay is 2000 mg/kg. For toxic substances, a high-dose level is selected to represent the maximum tolerated dose (MTD), defined as that dose demonstrating signs of bone marrow toxicity or other clear signs of systemic effects. The MTD is defined as a dose producing signs of toxicity such that higher dose level would be expected to produce mortality.

In the bone marrow metaphase analysis assay, dividing bone marrow cells are arrested in metaphase by an intraperitoneal injection of colchicine.⁶⁶ Metaphase cells are collected at two time points following dose administration. As in the *in vitro* assay, sample times are selected to assure analysis of first-division metaphase cells, both nondelayed and delayed. Cells are evaluated microscopically for chromosome aberrations as is done in the *in vitro* assay. Five animals per dose levels are typically included, although this is currently undergoing reevaluation during the updating of the OECD guideline.⁶⁷ Animals exposed to vehicle alone are used as negative controls and the spontaneous background rate is very low.

Historically, positive controls are routinely included in each assay, although this is being revised in recent guidelines with a movement to using previously prepared slides in order to avoid unnecessary animal usage. The most frequently encountered positive controls are cyclophosphamide or EMS. The purpose of this control group is to demonstrate an ability of the target cells to respond to chemical insult. Because of variation caused by uptake, distribution, and metabolism of the chemical and cell cycle kinetics of the target cells, the

magnitude of the positive response is greatly affected by the metaphase collection time. As in the *in vitro* systems, dose levels for positive controls should be selected to yield a moderate level of activity, thereby allowing for evaluation of the sensitivity of the test system.

For data analysis, the ratio of cells in mitosis to total number of cells (mitotic index) and the total number and types of aberrations should be presented for each animal. As with the *in vitro* assay systems, gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the average number of aberrations per cell.^{68,69} The percentage of damaged cells in the total population of cells scored is calculated for each treatment group. The severity of damage within the cells is reported as the average number of aberrations per cell for each treatment dose. Male and female animals should be analyzed separately.

As always, conclusions are based on sound scientific judgment; however, as a guide to interpretation of the data, the test article is considered to induce a positive response when the number of aberrant cells is significantly increased in a dose-responsive manner relative to the vehicle control. A significant increase at the high dose only with no dose response is considered equivocal. Similarly, a significant increase at one dose other than the high dose with no dose response is considered equivocal. The test article is judged negative if no statistically significant increases in percent aberrant cells are observed relative to the vehicle control group at any sampling time. An equivocal response should be clarified with additional testing if possible, using modified testing conditions.

IN VIVO BONE MARROW AND PERIPHERAL BLOOD MN

The *in vivo* MN test is often used in place of the technically more difficult and more laborious bone marrow metaphase assay. Since micronuclei are formed by intact chromosomes or chromatid fragments that are not incorporated into the nuclei of daughter cells during cell division, their presence is used to detect agents that either are clastogens or alter integrity or function of the spindle apparatus. Because of the strong correlation between chromosomal breakage and micronuclei formation, the two assay systems are considered to be equivalent for screening purposes.^{70,71} The MN test is routinely performed in rodents (rats and mice), and can be applied to other species including dogs and monkeys with appropriate validation. The *in vivo* MN assay is described in OECD Test Guideline 475.⁷²

The *in vivo* MN test can be applied to animals with acute treatment or integrated in subacute general toxicology studies such as 28 day repeat-dose rodent toxicity studies. During maturation in the bone marrow of hematopoietic cells from erythroblasts to erythrocytes, the nucleus is extruded forming polychromatic erythrocytes (PCEs). Over 24–48 h, PCEs mature into normochromatic erythrocytes (NCEs) and then migrate into the peripheral blood as reticulocytes (RETs). Micronuclei persist in the cytoplasm of the enucleated PCEs and NCEs in the bone marrow and RETs in the blood forming the basis for both the *in vivo* bone marrow and peripheral blood MN assays. The formation of micronucleated

PCEs (mnPCEs) requires one cell cycle of the nucleated precursor cell and the extrusion of the nucleus. Acute *in vivo* MN assays can be dosed one time and bone marrow collected for evaluation 24 and 48 h after dosing or dosed 24 h apart of two consecutive days and bone marrow evaluated 24 h after the last dose administration. Only nucleated precursor cells can give rise to test article-associated micronuclei. For this reason, microscopic scoring of PCEs and mnPCEs is done 24 h after the last dose administration to exclude cells that were already enucleated at the time of dosing. Because bone marrow toxicity can delay cell division, it is also important to determine the ratio of PCEs to NCEs in the bone marrow. A reduction in PCEs relative to NCEs is indicative of bone marrow toxicity.⁷³ The peripheral blood can be sampled at least 48–72 h after first exposure. A measurement of micronuclei peripheral blood erythrocytes can detect accumulated micronuclei in blood. One confounding factor is that in some species, the spleen gradually filters micronucleated RETs (mnRETs) for the peripheral blood. Splenic filtration of mnRETs can be overcome using techniques described later. Mice do not exhibit splenic filtration of mnRETs.

mnPCEs and mnRETs can be detected manually or using automated analysis (image analysis and flow cytometry when appropriately validated). Smears prepared for manual scoring will be stained with DNA-specific stain, example, acridine orange or non-DNA-specific stain, example, Giemsa (for mouse only), and are evaluated microscopically for mnPCEs. The bone marrow collected for flow cytometry analysis will be filtered through a cellulose column to eliminate the cells with nucleus. The proportion of PCEs or RETs among total erythrocytes is also measured as an indicator of bone marrow toxicity. The animal data will be considered too toxic to be evaluated if the proportion is <20% of concurrent vehicle control value in manual scoring, or <5%–10% measured using flow cytometry.

Positive controls were routinely included for every assay. There is a gradual trend to move away from the inclusion of positive control groups in every study. As in the bone marrow metaphase assay, the positive control when used is usually given by the same route as the test article. The positive control response is greatly influenced by the route of administration, which can affect uptake and distribution as well as optimum sampling time.⁷⁴ The positive control must be statistically elevated relative to the concurrent negative control for the test to be valid. As per the recent ICH S2 (R1) guideline,¹⁷ for *in vivo* studies, it is considered sufficient to treat animals with a positive control only periodically, and not concurrently with every assay, after a laboratory has established competence in the use of the assay. Options are available to randomly include previously prepared positive control bone marrow slides or positive controls blood samples as a scoring control.

For evaluation of the data, the incidence of mnPCE/mnRET should be presented for each animal and treatment group. Statistical significance is determined using appropriate statistical methods. All analyses should be performed separately for each sex. To quantify the test article effect on erythropoiesis, an indicator of bone marrow toxicity, the

proportion of PCE/RET to total erythrocytes should also be presented for each animal and treatment group. The historical control data for negative control (vehicle control) and positive control should be established by the laboratories routinely performing these assays to determine the validity of the study.

As a guide to interpretation of the data, the test article is considered to induce a positive response if a dose-responsive increase in mnPCE/mnRET is observed and one or more dose levels are statistically elevated relative to the vehicle control at any sampling time. If a single treatment group is significantly elevated at one sacrifice time with no evidence of a dose response, the assay is considered a suspect or unconfirmed positive, and a repeat experiment will be recommended. The test article is judged negative if no statistically significant increases in mnPCE above the concurrent vehicle control values are observed at any sampling time. Statistical significance should not be the only determination factor for a positive response. Biological relevance should be determined based on scientific judgment.

For both the bone marrow metaphase assay and the MN test, it is important to collect and analyze bone marrow samples at multiple time points.⁷⁵ To allow for metabolic activation and because many clastogens cause substantial cell cycle delay, no single sampling time is optimal. In the event of negative findings, it may be necessary to demonstrate that the target cells (i.e., bone marrow) were exposed to the test article. This may be achieved by a measure of bone marrow toxicity (mitotic inhibition or depressed PCE/total erythrocyte ratio), or in its absence, tissue distribution data. In some cases, blood serum levels may be sufficient to document exposure.

Micronuclei are also present in erythrocytes so can be measured in the peripheral blood. In some species such as rats, the spleen filters out circulating micronuclei.^{76,77} In this case, it is important to use the methods to analyze the newly formed RETs^{78,79} along with a sufficiently large sample size for increased statistical power to compensate for the lower MN levels in rat blood than in bone marrow.⁸⁰ Peripheral blood is ideal for MN analyses because of the relatively simple and nonlethal sample collection at multiple time points.⁸¹

The ICH S2 (R1) guideline¹⁷ suggests the use of appropriately validated automated analyses to examine greater number of cells for analysis. These systems include flow cytometry and automated image analysis. Commercial kits for flow MN analysis are advantageous due to their robust biological standards and antiplatelet antibodies to ensure intra- and interlaboratory reproducibility of data.⁸²

ANALYSIS OF CLASTOGENIC AND ANEUGENIC MODE OF ACTION IN THE MN ASSAY

Micronuclei can be formed by missegregation of a chromosome fragment that results from chromosome breakage and/or missegregation of an entire chromosome(s) as a result of damage to the mitotic apparatus. Since all normal chromosomes have a centromere associated with a kinetochore

structure, a MN that contains an entire chromosome(s) can be determined by a special staining technique described later for the presence of centromere DNA sequence or kinetochore protein within the MN. A chemical that induces the formation of micronuclei containing an entire chromosome(s) is considered to be an aneugen. In contrast, a MN that contains a chromosome fragment will have no staining for the presence of a centromeric sequence or kinetochore protein. A chemical that induces the formation of micronuclei containing chromosome fragments is considered to be a clastogen. Since an aneugenic mechanism of MN induction is a result of damage/dysfunction of the mitotic apparatus, which is a non-DNA target, aneuploidy is considered to have a threshold and may not represent a risk if human exposure is below the threshold.⁸³ Understanding the mode of action will play a role in the interpretation of a positive MN test result and the use of the data in risk assessment. Therefore, the investigation of the mode of action in MN formation is discussed in regulatory guidelines.

The most common methods for identification of the presence or absence of whole chromosomes in micronuclei are fluorescent *in situ* hybridization (FISH) and antikinetochore (calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, and telangiectasias syndrome [CREST]) techniques.^{84,85} FISH using a pan-centromeric probe or chromosome-specific probe is a powerful technique for localization of centromere DNA sequence within a MN.^{85,86} CREST using serum from CREST patients or commercial CREST antibodies can detect kinetochore proteins within a MN.^{84,87} The advantages and disadvantages and technical aspects of FISH and CREST methods in characterizing MN were reviewed.⁸⁸ In general, FISH probes that allow direct visualization of a centromere in the MN is more definitive than CREST that detects kinetochore proteins, but either method is acceptable.

FISH analysis is limited by the availability of centromere probes. Since commercial pan-centromere probes are currently available only for humans and mice, MN studies with FISH options are limited to human and mouse cells, example, MNvit assays using Human Peripheral Lymphocyte or TK6 cells and *in vivo* mouse bone marrow MN assay. The FISH staining process destroys the cell membrane and therefore the micronuclei that are analyzed cannot be visually identified with a specific cell.

CREST analysis can be applied to different species and can be applied in MNvit assays (isolated lymphocytes, CHO cells, TK6 cells, etc.) and *in vivo* bone marrow MN assays. A major drawback of CREST is that the kinetochore protein instead of centromere DNA is detected and there could be a false evaluation of aneugenic/clastogenic mode of action in MN formation if a test article inactivates or interferes with the formation of kinetochore protein.^{84,89} In addition, CREST analysis cannot distinguish between unique chromosomes and cannot detect nondisjunctional events,⁸⁹ but these analyses are largely research-oriented and not part of the standard MN analysis. Since the fixative (3:1 of methanol and acetic acid) regularly used in MNvit assay significantly reduces CREST staining intensity due to potential

destruction of kinetochore antigen recognition sites by acetic acid,⁹⁰ a cytospin technique is used in slide preparation of suspension cells.

The FISH/CREST scoring in MNvit assays with cyto-B technique is restricted to those binucleate cells with a minimum of 20 fluorescein isothiocyanate (FITC) staining signals within each nucleus counterstained with DAPI or PI.⁸⁹ The FISH/CREST scoring in *in vivo* bone marrow MN assays is restricted to MN counterstained with DAPI or PI, but cannot distinguish MN from PCEs and NCEs. Each FITC fluorescence signal from pan-centromere probes (FISH) or antibodies (CREST) conjugated with FITC represents one chromosome. A total of 100 micronuclei per treatment or animal are scored for centromere-positive MN (C+MN) or kinetochore-positive MN (K+MN). Concurrent aneugenic (e.g., vinblastine or colchicine) and clastogenic (e.g., MMC) positive controls are used to validate the assay.

Determination of an aneugenic or clastogenic mode of action is based on the proportion of C+MN or K+MN in chemically induced micronuclei. The potential impact of spontaneous micronuclei that contain about 50% C+(K+) MN, example, 30%–80% in human lymphocytes⁸⁵ or 50% in mouse bone marrow,⁹¹ may be ignored if the chemically induced MN frequency is at least threefold higher than that of vehicle control. As noted in guidelines,^{17,91} even potent tubulin poisons like colchicine and vinblastine do not produce 100% kinetochore-positive MN, but more typically 70%–80%. An aneugenic mode of action is determined if the majority (>70%) of chemically induced MN are centromere-positive.

IN VIVO ASSAYS FOR DNA DAMAGE, REPAIR, AND MUTATION

COMET ASSAY

Until recently, the most frequently used methods for the detection of DNA repair synthesis were unscheduled DNA synthesis (UDS) in individual cells or the detection of DNA single-strand break or strand breaks due to alkali labile sites in pooled cell populations using the alkaline elution assay. The UDS method is based on the replication of DNA during the excision repair of certain types of DNA lesions; as described in the earlier section, it involves the incorporation of tritiated thymidine into the DNA repair sites. While it provides information at the level of the individual cell, the methodology is difficult and requires the use of radioactivity. The assay is also considered to be not very sensitive.

The alkaline elution method is considered to be sensitive in the detection of DNA damage, but it has high variability and inconsistency. A more useful approach for assessing DNA damage is the comet assay. Östling and Johanson⁹² developed a microgel electrophoresis technique for detecting DNA damage at the level of the single cell. In their technique, cells embedded in agarose were placed on a microscope slide, the cells were lysed by detergents and high salt, and the liberated DNA was electrophoresed under neutral conditions. Cells with an

increased frequency of DNA double-strand breaks displayed increased migration of DNA toward the anode. The migrating DNA was quantified by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. The neutral conditions used greatly limited the general utility of the assay.

Subsequently, Singh et al.⁹³ introduced a microgel technique involving electrophoresis under alkaline (pH 13) conditions for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with increased levels of single-strand breaks, single-strand breaks associated with incomplete excision repair sites, and breaks induced by alkali-labile sites. Because almost all genotoxic agents induce more single-strand breaks and/or breaks induced by alkali-labile sites than double-strand breaks, this version of the assay offered increased sensitivity for identifying genotoxic agents. The comet assay (alkaline single cell gel electrophoresis assay) is a microgel electrophoretic technique that detects DNA damage in individual cells at pH \geq 13.

The purpose of this assay is to evaluate the genotoxic potential of the test article based upon its ability to induce DNA damage in selected organ cells of rats using the principals of the comet assay. In this assay, comet tail migration, % tail DNA (also known as % tail intensity), and tail moment are determined for each animal/treatment group and serve as parameters of DNA damage.

The comet assay is conducted according to established procedures^{94–99} and following protocol recommendations of the Japanese Center for the Validation of Alternative Methods (JaCVAM) for the detection of genotoxic carcinogens.¹⁰⁰

Animal handling and procedures should be performed according to Institutional Animal Care and Use Committees (IACUC) recommendations. Prior to starting the definitive assay, a dose range finding (DRF) test is performed to find the MTD. Usually, the animals are dosed with the same dosing regimen as planned for the definitive assay and animals are observed for clinical signs. The MTD is selected based on the results of the DRF. The MTD is defined as the dose that induces some signs of toxicity but is not expected to produce mortality within 2 or 3 days after administration, or severe and prolonged clinical signs of toxicity. The DRF is performed using both sexes; however, if there is no significant change in the toxicity profile, then only one sex is used in the definitive assay.

In the definitive assay, normally five groups of animals (rats or mice) are used. Three test article doses where the maximum dose is the MTD and two lower doses at one half and one quarter of the MTD are also included. Vehicle for the test article is used as the vehicle/negative control. There is one group of animals that is dosed with known DNA-damaging agent such as EMS or MMS at the concentration of 200 or 60 mg/kg, respectively. Since these are potent DNA-damaging agents, these require only one dose administration, 3–4 h prior to organ harvest.

The test article and vehicle control groups are dosed for either 2 days (dosed at 24 and 3 h prior to sacrifice) or 3 days (dosed at 48, 24, and 3 h prior to sacrifice). Table 13.2 is a study diagram that shows the most common dosing regimen.

It used to be a standard practice to dose one set of animals for short period (3–4 h) and another set for longer period (24 h) and then sacrifice at the end of the dosing period. However, repeat dosing with a single harvest 3 h after the last dose achieves the same purpose for short and long harvest periods.

At the time of organ harvest, it is a standard practice to save and freeze plasma and liver tissue for bioanalysis. Another piece of organ is saved in formalin for histopathology. Histopathology is usually performed if a significant increase in DNA damage is seen. This is to make sure that the DNA damage seen is not due to extreme toxicity in the tissue. Blood is saved to confirm exposure to the test article and the liver is saved for the metabolite analysis. Although liver and stomach or organs from the GI track are the most commonly used organs in the comet assay, the assay can easily be performed on any organ that can be used in making a single cell suspension. Following is a list of other common organs that can be used in the comet assay: liver, kidney, heart, lungs, stomach, duodenum, jejunum, ileum, colon, uterus, peripheral blood, skin, brain, spleen, and bone marrow.

All animals are euthanized 3–4 h after the last dose by CO₂ inhalation. Immediately following euthanasia, animals are dissected and the organs are removed and collected. A section of the organ is cut and placed in formalin for possible histopathology analysis.

A section of the organ is placed in chilled mincing solution (Hank's balanced salt solution [HBSS] with 20 mM ethylene diaminetetraacetic acid [EDTA], 10% DMSO, pH 7.0–7.5), then minced with fine scissors or scraped with a cell scraper to release the cells. Following organs are minced to release cells—liver, kidney, heart, lungs, uterus, brain, spleen, etc. Scraping to release cells is performed in the following organs—stomach, duodenum, jejunum, ileum, colon, etc. In order to make single cells from skin, a trypsinization process is used. The resulting cell suspension is strained into a prelabeled conical polypropylene tube through a cell strainer and used in slide preparation. Blood and bone marrow may be diluted with HBSS to prepare the optimal cell suspension for preparing slides.

Microscopic slides are prepared from the single cell suspensions prepared from each organ. An aliquot of cell suspension is mixed with low melting agarose and applied to microscopic slides previously coated with 1% normal melting agarose. The previously coated slides can also be purchased commercially. Once solidified, slides are submerged in a lysis solution to lyse the cells and nuclei. After cell lysis, slides are washed with neutralization buffer and placed in an electrophoresis chamber containing an alkaline buffer (pH > 13). The alkaline pH unwinds the DNA to single-stranded DNA. The slides are then electrophoresed. During electrophoresis, DNA fragments migrate in the direction of the electric current while high-molecular-weight undamaged DNA does not migrate. The slides are then electrophoresed. Following electrophoresis, the slides are removed and washed with neutralization buffer, dehydrated with 200-proof ethanol, air-dried, and stored at room temperature with desiccant for later scoring.

Prior to scoring, the slides are stained with an appropriate DNA stain. Usually, 50 randomly selected cells per slide are

scored resulting in a total of 100 cells animal. Several different computerized automated scoring systems are available commercially for scoring comet slides.

The following end points of DNA damage are assessed and measured (shown in Figure 13.1):

- Comet tail migration: Defined as the distance from the perimeter of the comet head to the last visible point in the tail
- % Tail DNA (also known as % tail intensity or % DNA in tail): Defined as the percentage of DNA fragments present in the tail
- Tail moment (also known as olive tail moment): Defined as the product of the amount of DNA in the tail and the tail length ($\% \text{ tail DNA} \times \text{tail length} \times 100$)¹⁰¹

Each slide is also examined for indications of cytotoxicity. The percentage of “clouds” is determined by scanning cells in each slide. The clouds also known as “hedgehogs,” are morphological indication of highly damaged cells often associated with severe genotoxicity, necrosis, or apoptosis. A cloud is produced when almost the entire cell DNA is in the tail of the comet and the head is reduced in size, almost nonexistent.¹⁰² Clouds with visible gaps between the nuclei and the comet tail are excluded from comet image analysis.

The DNA damage data (% tail DNA) in the vehicle control group must be within the historical vehicle control range, and the positive control group must be significantly increased relative to the concurrent vehicle control group ($p \leq 0.05$).

In order to quantify the test article effects on DNA damage, statistical analysis is usually performed on the data.^{103,104} The use of parametric or nonparametric statistical methods in evaluation of data is based on the variation between groups. The group variances for % tail DNA (or other parameters of DNA damage) generated for the vehicle and test article groups will be compared using Levene's test (significant level of $p \leq 0.05$). If the differences and variations between groups are found not to be significant, a parametric one-way ANOVA followed by a Dunnett post-hoc test is performed (significant level of $p < 0.05$). If Levene's test indicates heterogeneous group variances ($p \leq 0.05$), the suitability of a transformation of the original data is evaluated (e.g., using logarithm, or Box Cox transformed values of the original data) in an attempt to meet the normality criteria. Afterward, statistical analysis may be performed using the parametric tests described earlier. If parametric tests are not acceptable, nonparametric statistical methods (Kruskal Wallis or Mann Whitney test) may be used in evaluation of data. Linear regression analysis is used to determine a dose-response relationship ($p < 0.01$). Pair-wise comparison (Student's *t*-test, $p \leq 0.05$) is used to compare the data from the positive control group against the vehicle control group.

IN VIVO COMET AND MN ASSAYS—COMBINED AND INTEGRATED INTO TOXICOLOGY STUDIES

The comet assay can be easily combined with other *in vivo* assays such as a MN assay, a 14 day or a 28 day mammalian

TABLE 13.2

Study Design for *In Vivo* Comet Assay

Group	Treatment Dose Level (mg/kg)	Dose Administration	Animals/Sex
1	Vehicle	2 or 3 days	5
2	TA (low dose— $\frac{1}{4}$ of MTD)	2 or 3 days	5
3	TA (middle dose— $\frac{1}{2}$ of MTD)	2 or 3 days	5
4	TA (high dose—MTD)	2 or 3 days	5
5	Positive control	3 h prior to sacrifice	5

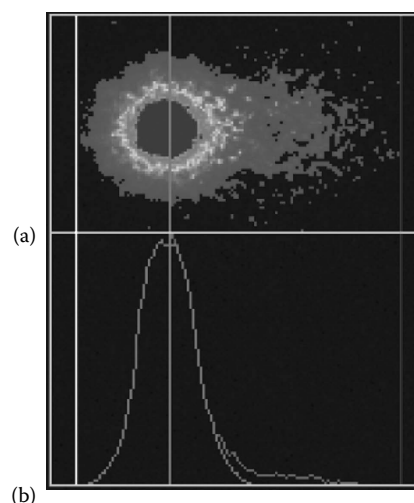


FIGURE 13.1 (See color insert.) (a) Image of genomic DNA from single cell used for Comet analysis; high molecular weight DNA remains in head on left and lower molecular weight DNA fragments migrate to right. Blue line on left marks start of genomic DNA, green line marks center of genomic DNA, and purple line on right marks furthest migration of DNA fragments. (b) Graphic representation of DNA density permitting quantification of DNA migration with genomic DNA in area covered by green curve and DNA fragments under the red curve.

toxicology study. When combining the comet assay with the MN assay, the dosing regimen is 3 days of dosing (48, 24, and 3 h prior to organ harvest). The positive control, EMS or MMS is dosed twice (24 and 3 h prior to organ harvest). Bone marrow is used for the MN assay using the slide method with manual scoring. If using the flow cytometric analysis for MN induction, the dosing regimen is increased by 1 day (72, 48, 24, and 3 h prior to organ harvest). Flow cytometric analysis can be performed with either bone marrow or peripheral blood. The details of MN analysis are provided in the section “*in vivo* bone marrow and peripheral blood MN assay.” When combining both the comet and MN assays with repeat-dose toxicology studies, the positive control is dosed as noted earlier depending on whether flow cytometric analysis of MN will be used. On the day of necropsy, organs are provided for the comet assay and bone marrow and/or blood is used for the MN assay.

IN VIVO TRANSGENIC RODENT MUTATION ASSAYS

Genetic toxicology routinely uses *in vitro* assays to detect mutations and chromosomal damage and *in vivo* assays to measure chromosomal damage for product safety testing. These assays are covered by OECD test guidelines and their use individually or in test batteries is described in various regulatory guidance documents for various product types such as the ICH S2 (R1) guideline for pharmaceuticals.¹⁷ Until recently, none of the *in vivo* assays with OECD test guidelines measure mutation *in vivo*. Because mutations are a key factor in somatic cell mutations leading to cancer and germ cell mutations leading to heritable mutations and birth defects, the lack of an accepted *in vivo* mutation assay has left a gap in the evaluation of hazard and risk for humans from chemicals with a mutagenic mode of action.

A recent advance in the area of *in vivo* mutation analysis is the finalization of OECD Test Guideline 488 titled *Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays*.¹⁰⁵ This guideline reflects the maturation of several transgenic rodent mutation (TRM) assays. The assays, literature, and data have undergone a detailed technical review in accordance with the OECD test guideline process,^{106–108} leading up to the adoption of a final TRM assay test guideline. The International Workshops on Genetic Toxicology (IWGT) has reviewed the TRM field, supported the OECD test guideline process for TRM assays, and developed a harmonized protocol for TRM assays.^{109,110} Since finalization of OECD Test Guideline 488, the European Chemicals Administration (ECHA) has begun to request a TRM assay as a follow-up to a positive *in vitro* mutation assay (Ames or mouse lymphoma large colony positive results) in a submission under REACH.

In vivo TRM assays fill a previously unmet need for an *in vivo* assay to help with the evaluation of positive *in vitro* genetic toxicology results where a mutagenic mode of action is suspected. In a regulatory setting, a TRM will likely be a second *in vivo* study complementing the initial *in vivo* MN assay that measures clastogenicity (DNA breakage) but not purely mutational events. The TRM assay will permit evaluation of tissue-specific mutagenic potential under *in vivo* exposure conditions modulated by mammalian adsorption, distribution, metabolism, and excretion. Many of the TRM assays were developed in the 1990s but never reached widespread acceptance for over 15 years. The availability of an OECD test guideline and the recent use of a TRM to investigate thresholds of mutagenesis and exposure to a potent alkylating agent in a pharmaceutical setting¹¹¹ have contributed to renewed interest and use of the models. TRM assays have also shown value in detecting induced mutagenesis in specific target organs due to high, local concentration of DNA-damaging agents, in an organ of first contact, or unique metabolism in a specific organ that otherwise is missed by other *in vivo* genetic toxicology assays.¹¹²

The most commonly used transgenic rodents for *in vivo* mutation analysis are the *lacZ* transgenic mouse (MutaTMMouse) and the Lambda LIZ *lacI* (BigBlueTM)

mouse and rat. Other TRM models have been developed including the *lacZ* plasmid mouse, gpt delta mouse and rat, rpsL, PhiX174, and sipF models. Use of a specific model has depended upon availability of animals, visibility of the model, and extent of validation.

All models follow similar general principles. A modified lambda phage or plasmid was microinjected into fertilized oocytes, reimplanted into a pseudo-pregnant female and founder animals created carrying the transgene. The transgene has the genetic sequence needed to record mutagenic events and then to report back the gene status as wild type or mutant. Multiple copies of the transgene are carried in every cell of each animal, permitting *in vivo* mutation analysis of almost any organ in the body.

Animals receive repeat-dose administrations using oral gavage or other routes as appropriate. Varying treatment durations and posttreatment sampling times have been used in the literature. From this literature it is apparent that each transgene appears to be neutrally carried in the cells without selection or enrichment. The observed mutant frequency following exposure to a mutagen increases in a linear fashion with the number of doses up to a point. The OECD detailed review papers and the IWGT harmonized protocol calls for 28 daily doses followed by tissue collection (sampling time) 3 days after the last dose administration. To investigate a putative mutagenic mode of action, only male animals are generally needed with group sizes of 5–10 animals per group. Animals are necropsied, somatic and germ cell tissues of interest removed, flash frozen, and stored at –70°C or lower for later mutation analysis. For mutant analysis, small samples of frozen tissue are removed, genomic DNA isolated, and the transgene recovered from the genomic DNA.

The recovered lambda phage can be repackaged into empty phage capsids creating infectious phage or the plasmid recircularized recreating the plasmid. With the appropriate *E. coli* host strain and culture conditions, the lambda phage can be adsorbed or plasmids electroporated into the bacteria, incubated, and mutant frequency determined. Several packaging or ligation and plating cycles may be needed and pooled to evaluate sufficient target genes for a single tissue. Early versions of the first TRM assays required visual screening of the color of wild type and mutant. Recent improvements permit the selective recovery of only mutants, thereby reducing time and costs for mutant screening.

MutaMouse was originally developed using *lacZ* of the *lac* operon as the target and reporter gene carried in the lambda shuttle vector. When packaged phage are adsorbed onto *E. coli* C (*lacZ*[–]) cells and plated in the presence of the chromagenic substrate X-Gal (a substrate for β -galactosidase that yields a blue product), wild-type phage give dark blue plaques and *lacZ* mutants give colorless or blue plaques with reduced color intensity.¹¹³ A subsequent modification was reported using an *E. coli* C (*galE-lacZ*[–]) cell and P-Gal medium.¹¹⁴ MutaMouse is available in a CD2F1 background (BALB/C \times DBA2).

The BigBlue mouse was created using a λ LIZ α shuttle vector, carrying portions of the *lac* operon responsible for

the regulatory control and synthesis of β -galactosidase. The transgene included the bacterial *lacI* repressor gene as a mutational target, together with the *lacO* operator, *lacP* promoter, and the carboxy-terminus portion of the *lacZ* reporter gene.^{115–117} When packaged phage are plated on *E. coli* SCS-8 cells in the presence of X-Gal, wild-type phage give colorless plaques and mutants give varying shades of blue plaques. No *lacZ* selective method is available. The BigBlue mouse is carried as a homozygote in C57BL6 mice. BigBlue mice are available as C57BL6 homozygous, C57BL6 heterozygous, and B6C3F1 heterozygous animals. BigBlue Fischer 344 rats are also available.

The original versions of MutaMouse and BigBlue were labor-intensive as they were based on nonselective mutation scoring requiring each packaged phage to be visually inspected. The lambda shuttle vector used in both mice contains the *cII* gene. The *cII* gene codes for a temperature-sensitive repressor protein that controls the lysogenic/lytic cycle of lambda phage. Mutations in the *cII* gene can be identified by plating packaged phage on *hfl*[–] *E. coli* and incubating at 24°C. Only *cII* mutants will enter a lytic cycle and give plaques at 24°C. Phage titer can be determined by incubation of separate plates at 37°C because both wild-type and mutant phage can enter a lytic cycle at 37°C and form plaques.¹¹⁸

IN VIVO PIG-A MUTATION ASSAY

The Pig-a assay is a relatively new *in vivo* forward gene mutation assay undergoing intensive research and validation. It relies on immunologic detection, by flow cytometry, of cells deficient in certain surface markers that are presumed to arise due to loss of glycosylphosphatidylinositol (GPI) anchor proteins. *Pig-a* gene product is involved in the first step of GPI anchor synthesis.¹¹⁹ Like the more familiar *hprt* gene, the *Pig-a* gene is X-linked and a single mutational event can lead to the mutant phenotype (the other genes involved in GPI anchor synthesis are autosomal, requiring two-hit kinetics to produce the mutant phenotype). Thus, loss of GPI-anchored surface markers is an acceptable surrogate for *Pig-a* gene mutation.^{120,121}

Use of Pig-a as a possible *in vivo* somatic mutation reporter gene was first proposed by Araten et al. based upon clinical studies of paroxysmal nocturnal hemoglobinuria.¹²² Development as an *in vivo* mutation assay in mice and rats began in earnest approximately a decade later.^{123–126} Shortly thereafter, a large-scale international validation effort was launched, using quantification of CD59-negative RETs and erythrocytes (RET^{CD59–} and RBC^{CD59–}, respectively) as a surrogate for *in vivo* *Pig-a* gene mutation in peripheral blood from rats. Following initial qualification (Stage II) trials using *N*-ethyl-*N*-nitrosourea (ENU),¹²⁷ each laboratory tested a prototypical mutagen previously studied at the reference lab (Stage III).^{128–133} The typical study design was a 28 day repeat-dose subchronic toxicity assay with three test article dose levels and concurrent vehicle control (all *n* = 5). Collectively, these studies demonstrated

the high transferability and exceptional reproducibility of the method, accumulation of mutant RET and RBC with repeated dosing, and applicability to multiple species. Many of the studies integrated multiple other genotoxicity end points during the 28 day treatment regimen (e.g., peripheral blood micronuclei, *ex vivo* chromosome aberrations, and/or comet) and/or at terminal sacrifice (all of those end points, as well as comet in assorted tissues). Other studies have applied similar methodology to other species and blood compartments.^{134–138} More recently, the use of immunomagnetic depletion of wild-type cells¹³⁹ and changes in study design (an increase in group size from five to six in the Stage IV studies) have greatly increased sample throughput and assay sensitivity and power.

The Pig-a assay offers three main benefits: use of small (<100 μ L) blood samples that allows repeat sampling and integration into standard toxicology studies; accumulation of mutant RET and RBC with repeat dosing, allowing detection and characterization of weak mutagens and low dose effects; and the ability to compare mutant induction across all species of toxicological concern, including man. For example, the assay has been used to quickly define dose–response relationships and identify thresholds for mutant induction by the direct-acting alkylating agents ENU and EMS.¹⁴⁰

At present, these analyses have largely been limited to rodent blood (peripheral, splenic, or bone marrow). Thus, as with all *in vivo* studies, demonstration of target tissue exposure is mandatory for a valid assay. In addition, the assay may suffer from a poor ability to detect agents that induce large deletions or chromosome rearrangements (as was found for the X-linked *hprt*). This possible deficiency could be addressed by integration of a complementary cytogenetic end point into the study design (notably, however, X-irradiation was found to be positive for induction of Pig-a mutant RET and RBC in mice¹⁴¹). Evaluation of more chemicals, including weak mutagens, negative compounds, and mutagen/nonmutagen pairs, is ongoing. Results from these studies will demonstrate the ultimate utility of the method.

IN VIVO/IN VITRO UDS ASSAY

In light of the change in the profile of hepatocyte metabolism immediately following removal of the cells from the animal,¹⁴² the *in vivo/in vitro* UDS assay is designed to account for complex patterns of metabolic activation, detoxification, uptake distribution, and excretion of chemicals. The experimental design is based on procedures as described by Mirsalis et al.¹⁴³ and Butterworth et al.¹⁴⁴ Hepatocytes are isolated at two time points (2–4 h and 12–16 h) following the administration (by gavage, intravenous injection, or intraperitoneal injection) of test article as well as positive and negative (solvent) controls. The collection of hepatocytes at two time points is an attempt to permit detection of maximum UDS response that occurs shortly after treatment such as with dimethylnitrosamine (DMN) or MMS as well as those

occur at 12–16 h posttreatment with DMN, 2-acetylaminofluorene (2-AAF), or 2,6-dinitrotoluene.

Primary rat hepatocytes are isolated from the liver by perfusion with ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) solution followed by collagenase solution. At 90–180 min after plating, the cells are washed once in serum-containing culture medium and refed with serum-free medium containing ^3H -TdR. After 4 h, the radioactive medium is removed and the plates were washed in serum-free medium plus 0.25 mM thymidine. The cells are then incubated for 17–20 h in culture medium plus 0.25 mM thymidine. After incubation, the cells are processed for autoradiography and silver grain counting according to procedures described for the *in vitro* UDS assay.

Commonly used positive controls include MMS and DMN for the 2–4 h time point, and DMN and 2-AAF for the 12–16 h time point. While 2-AAF can be used as the positive control via oral gavage, the level of UDS elicited is quite often very marginal. Because of its limited solubility in an aqueous vehicle, 2-AAF cannot be used as the positive control via the intravenous route. In light of these limitations, the authors' laboratory has conducted experiments and demonstrated that DMN can be used as the positive control (via oral gavage or intravenous injection) for both the early sampling time (2–4 h) and the late sampling time (12–16 h).¹⁴⁵ A substantial amount of the data in the published literature is based on chemicals (including chemicals commonly used as positive controls) administered by gavage (e.g., Mirsalis and Butterworth¹⁴⁶ and Ashby et al.¹⁴⁷). While the use of intraperitoneal injection is acceptable and considered more relevant in some situations, the optimal dose (for positive controls as well) may vary with the route of administration used. It is therefore important to consider the appropriate dose level for the positive control while selecting the route of administration.

For an assay to be considered valid, the proportion of cells in repair in the untreated and solvent-treated controls must be <15%, and the net nuclear grain count must be <1. In addition, the mean net nuclear grain count of the positive control must be at least 5 counts over that of the solvent control.

All conclusions are based on sound scientific judgment; however, the following is offered as a guide to interpretation of the data. Any mean net nuclear count that is increased by at least five counts over the solvent control is considered significant.^{49,50} A test article is judged positive if it induces a dose-related increase with no less than one dose significantly elevated above the solvent control. A significant increase in the mean net nuclear grain count in at least two successive doses in the absence of a dose response is also considered positive. A significant increase in the net nuclear grain count at one dose level without a dose response is judged equivocal. A test article is considered negative if no significant increase in the net nuclear grain counts is observed. The percentage of cells in repair (cells with ± 5 net nuclear grains) may also be used in making a final evaluation of the activity of the test article.

IN VITRO CELL TRANSFORMATION ASSAYS

The *in vitro* cell transformation assays are related to the induction of phenotypic alterations in cultured cells that are characteristic of tumorigenic cells.^{148,149} The cells go through morphological changes and the transformed cells with the characteristics of malignant cells have shown the ability to induce tumors in susceptible animals.^{150–152} These assays have been recommended for the prediction of carcinogenic potential of chemicals¹⁵³ and as an *in vitro* alternative to traditional 2 year mouse and rat or 6 month transgenic mouse carcinogenicity studies for testing of cosmetics in Europe.¹⁵⁴

The purpose of these studies is to assess the potential transforming activity of the test article(s) in cryopreserved Syrian hamster embryo (SHE) cells or in cell lines such as Balb/c 3T3¹⁴⁹ or Balb/c 3T3 cells that have been transfected with *v-Ha-ras*, called Bhas 42^{155–157} as measured by morphological transformation of the cells. The SHE cell transformation assay evaluates the phenotypic changes in the colonies which have originated from a single cell, whereas, in the Balb/c 3T3 and the newer version of Balb/c 3T3 using the Bhas 42 cells evaluates foci formation on top of the cell monolayer. This section describes the SHE and the Bhas 42 cell transformation assays.

Validation of the SHE and Balb/c 3T3 cell transformation assays (CTAs) was performed under the European Center for the Validation of the Alternative Methods (ECVAM). Now that the SHE CTA validation is complete, the OECD guidelines are in the process of being finalized. The CTA guideline will include both pH 6.7 and 7.0 variants of the SHE CTA.

SHE CELL TRANSFORMATION ASSAY

The assay is performed either at pH 6.7^{158–160} or at neutral pH 7.0.^{161,162} Prior to performing the definitive assay, a DRF assay is performed. Usually, 10 plates per concentration are treated starting at the maximum feasible concentration (lowest precipitating dose or 2000 $\mu\text{g}/\text{mL}$ or 10 mM whichever is lower). The concentration that has ~50% reduction in colonies based on the number, size, and density is used as the highest concentration in the definitive assay. If no toxicity is seen, then the maximum feasible concentration is used as the highest dose. The definitive assay is performed with forty 60 mm plates per dose.

Cryopreserved SHE cells (these can be purchased from vendors like BioReliance) from a tested and approved lot are thawed and grown to 50%–90% confluency in growth flasks (2–4 days). On day 1 of the assay, feeder cells are detached and suspended in culture medium in a growth flask on wet ice. The cells are x-ray irradiated to a point where they are still viable, yet no longer capable of replication (~5000 rad). Confirmation of this is made by preparing five dishes containing only feeder cells. Following irradiation, the cells are seeded at 2×10^4 cells/mL in culture medium and 2 mL of this suspension is placed into each 60 mm culture dish.

The day after seeding of the feeder cells, a second vial of SHE cells from the same lot as the feeder cells is thawed and seeded in a growth flask for 5 h. After the 5 h incubation period, the target cells are detached, counted, seeded in a concentration that should yield ~25–45 colonies/dish. For the target cell–adjusted dose groups, the required number of target cells per dish is determined from results of the cytotoxicity assay and the number of target cells is adjusted to yield 25–45 colonies. Dishes are incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a humidified atmosphere of $10\% \pm 0.5\% \text{CO}_2$ for ~24 h prior to test article treatment.

On day 3, the cells are treated with test article, vehicle, and positive controls and incubated for 24 h or 7 days depending on the dosing regimen in use. BaP at the concentrations of 1–10 $\mu\text{g/mL}$ is used as the positive control. For the 24 h dosing regimen, the test article is removed from the dishes and the cells are refed with culture medium and the plates are incubated for additional 6 or 7 days. Seven day dosing regimen requires the dishes with test article to be incubated for 7 days. At the end of the incubation period, the cells are fixed with methanol, stained, and coded to remove scorer's bias.

Each colony is evaluated and recorded as either normal or morphologically transformed (MT). Normal colonies contain cells with an organized, often flowing, pattern of growth with minimal cell crisscrossing, particularly where the cells are at a confluent density. Normal colonies also tend to be in a monolayer. MT colonies contain cells arrayed in an extensive randomly oriented, three-dimensional, stacked growth pattern, with crisscrossing of cells at the perimeter and in the interior of the colony. Cells in MT colonies frequently are more basophilic than their normal counterparts and have increased nuclear/cytoplasmic ratios.^{162,163}

The test article is evaluated according to the increase of MT colonies in the test article–treated dishes. Biological relevance is taken into account when evaluating as well as the statistically significant increase.

BHAS 42 CELL TRANSFORMATION ASSAY

Bhas 42 cells are v-Ha-*ras*-transfected Balb/c 3T3 clone A31-1-1 cells.

As in any other *in vitro* assay, a DRF test is performed and in the initiator transformation assay ~50% cytotoxic concentration or if nontoxic then maximum feasible dose is used as the maximum concentration. In the promoter assay, usually the dose that shows increase in growth or the maximum feasible dose is used as the highest concentration.

Initiator transformation assay: Cells are seeded in the six-well plates at 4000 cells/plate and grown for 24 h and treated with test article on day 1, then on day 4 the test article is removed and the plates are refed with culture media and incubated. One six-well plate per dose is used for the main

assay along with three wells of the six-well plate for the parallel toxicity determination. These plates are again refed on days 7, 10, and 14. Again, they are incubated undisturbed from days 14 to 21. The wells used for parallel toxicity determination are fixed on day 7. Vehicle and positive controls are also included in the assay; 3-methylcholanthrene (MCA) at 1.0 $\mu\text{g/mL}$ is used as the positive control in the initiator transformation assay.

Promoter transformation assay: Cells are seeded in the six-well plates at 14,000 cells/plate and grown for 3 days and treated with test article on day 4, then on days 7 and 10. On these days, the plates are refed with test article containing media. One six-well plate per dose is used for the main assay along with three wells of the six-well plate for the parallel toxicity determination. On day 14, the test article–containing media is removed and the plates are refed with culture media and the plates are incubated until day 21. Vehicle and positive controls are also included in the assay; 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at 50 ng/mL is used as the positive control in the promoter transformation assay.

On day 21, the plates are fixed, stained, and then scored for the number of transformed foci in each well and dose.

Transformed foci have the following morphological characteristics: (1) there are more than 100 cells in the foci, (2) spindle-shaped cells inside the foci are different in appearance from the contact-inhibited monolayer cells, (3) cells in the foci are more basophilic and stain darker than surrounding cells, (4) random orientation of cells at the edge of foci (crisscrossing), (5) dense multilayering of cells (piling up), and (6) foci show invasive growth into the monolayer of surrounding contact-inhibited cells. There are transformed foci not prominent in some of these characteristics.

Test article is considered to be a cell-transforming agent if statistically significant increases in the number of foci in the test article–treated doses are seen.

SCREENING ASSAYS

Early identification of potential genotoxic issues with candidate compounds is an essential part of a product development process. There are a variety of rapid, low-cost genetic toxicology assays that can be performed with milligram quantities of test article. These non-GLP-compliant assays are utilized early in the development process for various reasons, including lead optimization, prediction of the results of GLP regulatory-compliant assays, to investigate mechanism of action and to assess relative hazard or potency.

Selection of the appropriate assay or group of assays is critical to the success of a screening program. Many screening assays are available to investigate different mechanisms of DNA damage, each with different strengths and weaknesses. Some assays use the same cells and end points as used in core GLP regulatory assays that eventually will be run on the final drug or chemical. Other assays use different cells, end points, or biomarkers of DNA damage.

When designing a screening program, various factors need to be considered, including what the purpose of the testing is (e.g., prediction of GLP assays or investigation of mechanism of action), how the data will be used, the quantity of test article available, cost, and timeline. For example, screening for lead optimization for pharmaceuticals may only need a top dose of 1 mM following ICH S2 (R1) if predictivity of regulatory assay results is the goal while higher dose levels may be appropriate for industrial or agricultural chemicals.

The bacterial reverse mutation (Ames) screening assay measures a chemical's ability to induce reverse mutations in different strains of bacteria. The predictivity of a positive result in the Ames assay for genotoxic rodent carcinogenicity is very high and as a result, an Ames screening assay is often a key part of a genetic toxicology screening program. A number of miniaturized screening versions of the Ames assay are available including abbreviated versions of the standard assay using 100 mm culture dishes, 6-well cluster dishes, and 24-well cluster dishes. Test article requirements for these screening assays range from 5 to 120 mg, whereas a significant reduction in 900 mg to 1 g required for a standard OECD-compliant Ames assay.

Another modification of the standard Ames assay is the Ames II assay. The Ames II assay is a second-generation bacterial reverse mutation assay developed as a predictive screening assay for genotoxicity developed in Dr. Bruce Ames' laboratory at the University of California. The assay has been reported to have an 87% concordance to the standard Ames assay.¹⁶⁴ Like the standard Ames assay, the Ames II assay can detect both frameshift and base-pair substitutions under both nonactivation and exogenous (S9) metabolic activation conditions. Frameshift mutations are detected using the traditional TA98 *Salmonella* strain. The different types of base-pair substitutions are detected utilizing six *S. typhimurium* strains specifically engineered for the assay. Each strain carries a different missense mutation in the histidine operon that is designed to revert uniquely to one of the six possible base substitution combinations causing transitions or transversions in base sequence. The six strains are combined into a single culture called TAMix. The TAMix strains have a lower spontaneous reversion frequency compared to the standard *Salmonella* tester strains which makes it easier to utilize a microplate fluctuation format. The Ames II assay requires 6 mg or less of test article.

In vitro mammalian cell cytogenetic screening assays include miniaturized screening versions of GLP MNvit and chromosome aberration assays are available to assess the clastogenic potential of test articles. Assay versions with various cell types are used including HPBL, human TK6 (both offer the advantage of normal human p53 function), and CHO cells. With the recent adoption of the ICH S2 (R1) guideline,¹⁷ the MNvit assay has been added to the core battery of acceptable cytogenetic assays. The screening version of this assay predicts the standard MNvit assay described in the new OECD guideline 487. This assay can be scored either

microscopically or using flow cytometry as was described earlier in the *in vitro* cytogenetics section. The screening *in vitro* chromosome aberration assay uses the same cells and end points as used in regulatory GLP assays. Screening *in vitro* chromosome aberration assay designs usually eliminate pretoxicity tests and provide a summary of damage. Test article quantities for these assays are ~50 mg for *in vitro* flow MN assays and 150 mg for the microscopic scored versions of the assays.

The screening version of the mouse lymphoma assay predicts the standard assay, OECD 476. This assay measures forward mutations due to mutagenic or clastogenic mechanism. Elimination of pretoxicity testing, single cultures, and widely spaced doses are used. The screening version of the assay may be run in either the original soft agar selection format or the 96-well liquid selection format. Test article amounts for a screening mouse lymphoma assay are ~400 mg. Due to the nature of dosing, mutant expression, and mutant selection, *in vitro* HGPRT (CHO, V79, or CHL) are seldom performed.

Other *in vitro* screening assays are available to evaluate different genetic toxicology end points or to meet specific testing objectives. Some assays such as the *in vitro* comet or GreenScreen assay measure DNA damage that is "upstream" of the end points of mutation and cytogenetic damage measured in regulatory GLP assays, while other assays such as the Bhas 42 assay and SHE Cell Transformation assay can be used in a screening mode to evaluate a test article's ability to transform a "normal" cell into a "transformed" cancerous cell.

REGULATORY GUIDELINES

US FDA

US FDA Pharmaceuticals (CDER and Center for Biologics Evaluation and Research [CBER])

The ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use has developed guidelines for a number of disciplines to bring global harmony to safety testing of human drugs. The latest update of the ICH adopted Harmonised Tripartite Guideline S2 (R1), entitled "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use" June 2012 is available online.¹⁷ This guideline replaces previous guidelines, "Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals" (ICH Topic S2A, 1995) and "Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals" (ICH Topic S2B, 1997) which listed the following as required assays:

- A test for gene mutations in bacteria
- An *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma *tk* assay
- An *in vivo* test for chromosomal damage using rodent hematopoietic cells

The “Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use” guideline gives explicit guidance for selection of genotoxicity tests that will support the registration of a new pharmaceutical worldwide. There are two test battery options:

Option 1

- A test for gene mutation in bacteria (i.e., Ames/*E. coli* assay)
- A cytogenetic test for chromosomal damage (*in vitro* metaphase chromosome aberration test or MNvit test) or *in vitro* mouse lymphoma *tk* gene mutation assay
- *In vivo* test for genotoxicity, generally a test for chromosome damage using rodent hematopoietic cells, either for micronuclei or for chromosome aberrations in metaphase cells

Option 2

- A test for gene mutation in bacteria (i.e., Ames/*E. coli* assay)
- *In vivo* test for genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second *in vivo* assay. Typically, this would be a DNA strand breakage assay in liver, unless otherwise justified

Some important differences in these revised guidelines compared to the previous ICH guidelines are shown as follows:

1. In both options, the *in vivo* assays can be conducted using acute dosing or repeat dosing and some end points can be integrated into repeat-dose studies (e.g., micronuclei).
2. Specific guidance on the appropriate high dose for repeat-dose studies includes
 - Maximum feasible dose from repeat-dose study is similar to the maximum dose in an acute dosing study
 - Limit dose of 1000 mg/kg for 14 days or longer
 - Established plateau/saturation of exposure
 - Fifty percent or higher of acute top dose
 - In test Option 1, if the *in vitro* mammalian cell test is negative and a MN end point is integrated into a multiple administration toxicology study, the doses are generally considered appropriate when the toxicology study meets the criteria for an adequate study to support human clinical trials.
3. The assessment of micronuclei can be by flow cytometry including cells from rat or mouse, blood or bone marrow.
4. A confirmatory assay for bacterial mutation is not required if there is a clear positive or clear negative

result. The plate incorporation or the preincubation method is appropriate.

5. Positive controls

- There is no need for concurrent positive controls for *in vivo* studies after laboratories have established competence. For instance, existing coded slides from positive control-treated animals can be used for *in vivo* cytogenetic assays. Positive controls for the comet assay are advised since the assay is relatively new for regulatory submissions.
- For the *in vitro* mammalian cell assays, positive controls with metabolic activation only are sufficient if done at the same time as the nonactivated assay.

6. Top concentrations for *in vitro* mammalian cell assays:

- 1 mM or 0.5 mg/mL whichever is lower when not limited by solubility (previously the top concentration was 10 mM or 5 mg/mL)
- For pharmaceuticals with unusually low molecular weight (<200), higher test concentrations should be considered
- The top concentration for compounds with limited solubility is the lowest precipitating concentration unless toxic at a lower dose. This is different from previous guidelines that required testing at concentrations above precipitation to achieve toxicity limits (50% in the *in vitro* cytogenetic assays, 80%–90% in the *in vitro* mouse lymphoma *tk* gene mutation assay)

7. The appropriate measure of toxicity for *in vitro* cytogenetic assays is relative cell growth.

Compounds giving positive results in the standard test battery may, depending on their therapeutic use, need to be tested more extensively. Specific cases are discussed in the guideline.

The guideline gives examples of situations in the test batteries described earlier may need modification. These are summarized as follows:

- If the test material is highly toxic to bacteria, as might be expected with an antibiotic, the bacterial mutation assay should still be conducted but one of the *in vitro* mammalian cell assays should also be conducted (Option 1 should be followed).
- Compounds with specific structural alerts like azo structures require protocol modifications.
- For certain compounds, standard *in vivo* tests may be inappropriate, such compounds that are not systemically absorbed and do not reach the target tissue.

US FDA Food Additives (CFSAN)

The FDA Center for Food Safety and Applied Nutrition (CFSAN) has published Toxicological Principles for the

Safety Assessment of Direct Food Additives and Color Additives Used in Food (Redbook).⁹¹ In Chapter IV C1, Short-Term Tests for Genetic Toxicity, the agency recommends a battery consisting of a test for gene mutations in bacteria, an *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells or an *in vitro* mouse lymphoma thymidine kinase^{+/−} gene mutation assay, and an *in vivo* test for chromosomal damage using mammalian hematopoietic cells. CFSAN does note that they prefer the use of the mouse lymphoma tk^{+/−} assay as this assay measures heritable genetic damage arising by several mechanisms in living cells and is capable of detecting chemicals that induce either gene mutations or heritable chromosomal events, including genetic events associated with carcinogenesis. CFSAN also notes that in performing the mouse lymphoma tk^{+/−} assay, either the soft agar or the microwell method is acceptable.

US FDA Medical Devices (CDRH)

The Center for Devices and Radiological Health (CDRH), based on General Memorandum #95-1, has indicated that ISO 10993 Part 1 “Biological Evaluation of Medical Devices”¹⁶⁵ would replace the Tripartite Biocompatibility Guidance, which had been used previously. The ISO guideline 10993 Part 3 “Tests for genotoxicity, carcinogenicity and reproductive toxicity”¹⁶⁶ indicates that there are two options for genotoxicity testing. The first option is to perform a test for gene mutations in bacteria, a test for gene mutations in mammalian cells, and a test for clastogenicity in mammalian cells to be performed per OECD guidelines 471, 476, and 473, respectively. The second option is to perform a test for gene mutations in bacteria and a test for gene mutations in mammalian cells, specifically a mouse lymphoma assay incorporating colony number and size determination in order to cover both end points (clastogenicity and gene mutations). If the results of all *in vitro* tests are negative, further genotoxicity testing

in animals is not normally indicated. If any of the *in vitro* tests is positive, an *in vivo* test should be chosen on the basis of the most appropriate end point identified by the *in vitro* tests.

US FDA Biologics (CBER)

The ICH S6 (R1) guidance (Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals) indicates that genotoxicity studies are not applicable to biotechnology-derived pharmaceuticals unless there is a cause for concern about the product such as the presence of an organic linker molecule in a conjugated protein product. For this reason, genetic toxicology testing would only be required on a case-by-case basis. In general, the farther removed a biological product is from an endogenous material, the more likely it is that testing will be required.¹⁶⁷

EPA-REGULATED PRODUCTS

New Chemicals

EPA-regulated materials requiring mutagenicity testing are covered under either the Toxic Substances Control Act (TSCA) or the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). TSCA regulates new chemicals (TSCA Section 5) and those that were already in commerce at the time the act was passed (TSCA Section 4). Although TSCA does not require toxicology testing for submission of a Pre-Manufacture Notice (PMN), the agency has indicated that if the material in question meets certain criteria relating to human exposure or to release into the environment then a battery of safety tests should be performed. For the genetic toxicology component, the test battery is a Bacterial Reverse Mutation test (US EPA OPPTS 870.5100¹⁶⁸, OECD 471¹⁶) or a Mammalian erythrocyte MN (intraperitoneal route) (OPPTS 870.5395¹⁶⁹, OECD 474⁷²) (Table 13.3).

TABLE 13.3

Criteria Triggering Toxic Substances Control Act Pre-Manufacture Notice Test Battery

1. More than 1000 workers exposed
2. More than 100 workers exposed by inhalation to more than 10 mg/kg/day
3. More than 100 workers exposed by inhalation to 1–10 mg/day for more than 100 days per year
4. More than 250 workers exposed by routine dermal contact for more than 100 days per year
5. Presence of the chemical in any consumer product where (a) the physical state of the chemical in the product and (b) the manner of use would make exposures likely
6. More than 70 mg/year exposure via surface water
7. More than 70 mg/year of exposure via air
8. More than 70 mg/year exposure via groundwater
9. More than 10,000 kg/year release to environmental media
10. More than 1000 kg/year release to surface water after calculated estimates of treatment

Existing Chemicals

Toxicology testing for existing chemicals is administered through test rules or consent orders. Because Section 4 chemicals often have wide distribution in the environment and/or widespread human exposure, the basic test battery has, in addition to the bacterial reverse mutation test and the *in vivo* MN test, an *in vitro* gene mutation assay in mammalian cells. Positive responses in the two gene mutation assays trigger a study for “interaction with gonadal DNA.” The category includes end points such as SCE, alkaline elution, and UDS. Positive evidence of interaction with gonadal DNA triggers a specific locus test, either the visible or biochemical assay. A positive response in an *in vivo* bone marrow cytogenetics assay triggers a dominant lethal assay, and a positive response in a dominant lethal triggers a heritable translocation assay. Chronic studies for carcinogenicity are triggered by positive responses in the *in vivo* MN assay and either of the *in vitro* assays. Single positive responses or positive responses in the two *in vitro* assays result in a “data review.”¹⁷⁰

Agricultural Chemicals

The Office of Pesticide Programs (OPP) mutagenicity testing scheme is similar to the TSCA requirements. The test battery consists of a bacterial reverse mutation assay, a mouse lymphoma gene mutation assay, and an *in vivo* bone marrow cytogenetics assay, either metaphase or MN. If one chooses the CHO or V79 HGPRT gene mutation assay in place of mouse lymphoma, OPP then requires an additional assay for *in vitro* chromosomal aberrations. Study designs are indicated in the EPA Harmonized Test Guidelines Series 870—Health Effects Test Guidelines. In September 2012, the OPP issued a guidance on “Advances in Genetic Toxicology and Integration of *in vivo* Testing into Standard Repeat Dose Studies” in which they indicated acceptance of several of the ICH S2 (R1) changes.¹⁷¹ The guidance noted that OPP would be accepting data from MNvit assays (OECD test guideline 487⁵⁸), and that OPP has endorsed the new OECD test guideline (488) for the transgenic rodent gene mutation assay.¹⁰⁵ Also, they encourage the incorporation of genotoxicity end points into routine toxicology studies where scientifically feasible. OPP also indicated that the MN and comet assays can effectively be incorporated into routine toxicology studies. In addition, although the *Pig-a* gene mutation assay does not have an OECD Test guideline, OPP noted that it was a “promising new *in vivo* mutation test that is sensitive and less costly than the transgenic rodent gene mutation assay, and can be integrated into repeat-dose standard toxicology tests.”

JAPANESE REGULATORY AGENCIES

Pharmaceuticals and Workplace Chemicals

The Ministry of Health, Labour and Welfare (MHLW)¹⁷² was formed by the merger of the former Ministry of Health and Welfare (regulating pharmaceuticals and standards for foods, food additives, etc.) and the Ministry of Labor (regulating registration of new chemical substances, marketing of chemicals,

raw materials, intermediates, by-products, and waste generated in the workplace). Japan is a signatory to the ICH guidelines and therefore, for pharmaceuticals for human use, follows the ICH S2 (R1) guidance described previously.¹⁷ A dated, but still useful review of genetic toxicology regulations in Japan was written by Sofuni.¹⁷³ For workplace chemicals, only the bacterial reverse mutation assay is required. If the bacterial assay shows “strong mutagenicity potential,” an *in vitro* chromosomal aberration assay will be additionally required.

Agricultural Chemicals

The Ministry of Agriculture, Forestry and Fisheries (MAFF)¹⁷⁴ requires three major end points to be analyzed on agricultural chemicals. These include reverse mutation in bacteria, *in vitro* chromosomal aberrations, and an *in vivo* MN assay.

New Chemicals

The Ministry of Health, Labor and Welfare, Ministry of Economy, Trade and Industry (METI),¹⁷⁵ replacing the Ministry of International Trade and Industry, and the Agency of Environment, published guidelines for toxicity testing of new chemicals. Two mutagenicity studies are required and include a reverse mutation assay in bacteria and chromosomal aberrations in cultured mammalian cells. A mouse MN is also specified as one of the seven additional toxicity screening tests.

EUROPEAN ECONOMIC COMMUNITY

Pharmaceuticals

Negative results in a bacterial reverse mutagenicity assay alone, or in combination with an *in vitro* cytogenetics assay, is generally sufficient to begin clinical trials. To receive a product license, the EEC Committee on Proprietary Medicinal Products (CPMP)¹⁷⁶ follows the ICH guidelines described previously.¹⁷

Pesticides and Chemicals

REACH is the European Community Regulation on chemicals and their safe use.¹⁷⁷ This legislation deals with the registration, evaluation, authorization, and restriction of chemical substances and took effect on June 1, 2007. This law requires companies manufacturing or importing chemical substances into the European Union in quantities of 1 ton or more per year to register these substances with a European Chemicals Agency (ECHA).

ECHA has set three major deadlines for registration of chemicals. In general, these are determined by the amount manufactured or imported, as noted as follows.

Requirements vary, depending on tonnage:

- ≥1 ton—Annex V—registered by May 31, 2018
- ≥10 tons—Annexes V and VI—registered by May 31, 2018
- ≥100 tons—Annexes V–VII—registered by May 31, 2013
- ≥1000 tons—Annexes V–VIII—registered by November 30, 2010

According to REACH, mutagenicity studies are required for all the tonnage bands: Annexes V, VI, and VIII. Unlike most other end points, a negative mutagenicity result *in vitro* can be considered sufficient evidence for nonmutagenic potential but positive results must be confirmed *in vivo*. At 1–10 ton level (Annex V), the *in vitro* gene mutation study in bacteria (Ames test) is required. At Annex VI level (10–100 tons), an additional two *in vitro* studies are required: a cytogenicity study and a gene mutation study in mammalian cells. If there are any positive results within these *in vitro* tests, *in vivo* mutagenicity studies are required. At higher tonnage, *in vivo* studies are needed. REACH does not state which *in vivo* tests you need to carry out. However, it would be reasonable to carry out the *in vivo* versions of the test(s) which proved positive at *in vitro* level. Test designs should follow the OECD test guidelines.

REFERENCES

1. Valerio, L.G., *In silico* toxicology models and databases as FDA Critical Path Initiative toolkits, *Human Genom.*, 5, 200–207, 2011.
2. FDA (US Food and Drug Administration), Draft Guidance for Industry, Genotoxic and carcinogenic impurities in drug substances and products: Recommended approaches, US Food and Drug Administration, Washington, DC, 2008. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM079235.pdf (accessed October 24, 2012).
3. ICH (International Conference on Harmonisation), ICH Test Guideline M7: Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk, Endorsed at Step 1 of the ICH process, June 9, 2010. <http://www.ich.org/products/guidelines/multidisciplinary/article/multidisciplinary-guidelines.html> (accessed October 24, 2012).
4. Kavlock, R.J., Ankley, G., Blancato, J., Breen, M., Conolly, R., Dix, D., Houck, K. et al., Computational toxicology—A state of the science mini review, *Toxicol. Sci.*, 103, 14–27, 2008.
5. Lahl, U. and Gundert-Remy, U., The use of (Q)SAR methods in the context of REACH, *Toxicol. Mech. Meth.*, 18, 149–158, 2008.
6. OECD (Organisation of Economic Co-operation and Development), The OECD QSAR toolbox for grouping chemicals into categories, OECD, Paris, France, 2012. <http://www.qsartoolbox.org/project.html> (accessed November 1, 2012).
7. Ashby, J. and Tennant R.W., Chemical structure, *Salmonella* mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP, *Mutat. Res.*, 204(1), 17–115, 1988.
8. Matthews, E.J., Kruhlak, N.L., Benz, R.D., Contrera, J.F., Marchant, C.A., and Yang, C., Combined use of MC4PC, MDL-QSAR, Leadscape PDM, and Derek for Windows Software to achieve high-performance, high-confidence, mode of action-based predictions of chemical carcinogenesis in rodents, *Toxicol. Mech. Methods*, 18, 189–206, 2008.
9. Mahadevan, B., Snyder, R.D., Waters, M.D., Benz, R.D., Kemper, R.A., Tice, R.R., and Richard, A.M., Genetic toxicology in the 21st century: Reflections and future directions, *Environ. Mol. Mutagen.*, 52(5), 339–354, 2011.
10. Contrera, J.F., Improved *in silico* prediction of carcinogenic potency (TD50) and the risk specific dose (RSD) adjusted Threshold of Toxicological Concern (TTC) for genotoxic chemicals and pharmaceutical impurities, *Regul. Toxicol. Pharmacol.*, 59(1), 133–141, 2011.
11. McCann, J., Choi, E., Yamasaki, E., and Ames, B.N., Detection of carcinogens as mutagens in the *Salmonella* microsome test: Assay of 300 chemicals, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 5135, 1975.
12. Sugimura, T., Yahagi, T., Nagao, M., Takeuchi, M., Kawachi, T., Hara, K., Yamasaki, E., Matsushima, T., Hashimoto, Y., and Okada, M., Validity of mutagenicity tests using microbes as a rapid screening method for environmental carcinogens, in *Screening Tests in Chemical Carcinogenesis*, Montesano, R., Bartsch, H., and Tomatis, L., eds. IARC Scientific Publications No. 12, Lyon, France, p. 81, 1976.
13. Dunkel, V.C., Collaborative studies on the *Salmonella* microsome mutagenicity assay, *J. Assoc. Off. Anal. Chem.*, 62, 874, 1979.
14. Dunkel, V.C., Zeiger, E., Brusick, D., McCoy, E., McGregor, D., Mortelmans, K., Rosenkranz, H.S., and Simmon, V.F., Reproducibility of microbial mutagenicity assays: I. Tests with *Salmonella typhimurium* and *Escherichia coli* using a standardized protocol, *Environ. Mutagen.*, 6(Suppl 2), 1, 1984.
15. Dunkel, V.C., Zeiger, E., Brusick, D., McCoy, E., McGregor, D., Mortelmans, K., Rosenkranz, H.S., and Simmon, V.F., Reproducibility of microbial mutagenicity assays: II. Testing of carcinogens and non-carcinogens in *Salmonella typhimurium* and *Escherichia coli*, *Environ. Mutagen.*, 7(Suppl 5), 1–248, 1985.
16. OECD (Organisation of Economic Co-operation and Development), Test Guideline 471, OECD Guideline for testing of chemicals—Bacterial reverse mutation assay. OECD, Paris, France, adopted July 21, 1997. <http://www.oecd.org/chemicalsafety/assessmentofchemicals/1948418.pdf> (accessed November 2, 2012).
17. ICH (International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guideline. S2 (R1) document recommended for adoption at step 4 of the ICH process on 9 November 2011. Adopted at Step 5 in Europe by CHMP December 2011 (issued as EMA/CHMP/ICH/126642/2008). Adopted at Step 5 in US by FDA on June 7, 2012 (issued as Vol. 77, No. 110, pp. 33748–33749). Adopted in Japan at Step 5 September 20, 2012 (issued as PFSB/ELD Notification No. 0920-2), http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2R1_Step4.pdf (accessed 2 November 2012).
18. Wilcox, P., Naidoo, A., Wedd, D.J., and Gatehouse, D.G., Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains, *Mutagenesis*, 5, 285, 1990.
19. Maron, D.M. and Ames, B.N., Revised methods for the *Salmonella* mutagenicity test, *Mutat. Res.*, 113, 173–215, 1983.
20. Ames, B.N., McCann, J., and Yamasaki, E., Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test, *Mutat. Res.*, 31, 347, 1975.
21. Eisenstadt, E., Miller, J.K., Kahng, L.-S., and Barnes, W.M., Influence of *uvrB* and pKM101 on the spectrum of spontaneous UV- and γ -ray induced base substitutions that revert hisG46 and *Salmonella typhimurium*, *Mutat. Res.*, 220, 113, 1989.
22. McCann, J., Springarn, N.E., Kobori, J., and Ames, B.N., Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 979–983, 1975.
23. Walker, G.C., Plasmid (pKM101)-mediated enhancement of repair and mutagenesis: Dependence on chromosomal genes in *Escherichia coli* K12, *Mol. Gen. Genet.*, 152, 93, 1977.

24. Shanabruch, W.G. and Walker, G.C., Localization of the plasmid (pKM101) gene(s) involved in rec A+ lex A+ dependent mutagenesis, *Mol. Gen. Genet.*, 179, 289, 1980.
25. Langer, P.J., Shanabruch, W.G., and Walker, G.C., Functional organization of plasmid pKM101, *J. Bacteriol.*, 145, 1310, 1981.
26. Ames, B.N., Lee, F.D., and Durston, W.E., An improved bacterial test system for the detection and classification of mutagens and carcinogens, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 782, 1973.
27. Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A., and Ames, B.N., A new *Salmonella* tester strain (TA102) with A-T base pairs at the site of mutation detects oxidative mutagens, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 7445–7449, 1982.
28. Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D., Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 2281, 1973.
29. Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., and Okada, M., Mutagenicities of *N*-nitrosamines on *Salmonella*, *Mutat. Res.*, 48, 121, 1977.
30. Prival, M.J. and Mitchell, V.D., Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S9, *Mutat. Res.*, 97, 103, 1982.
31. Matsushima, T., Genotoxicity of new Japanese chemicals, in *Mutation and the Environment*, Part E. Wiley-Liss, New York, 251 p., 1990.
32. Dakoulas, E.W., Hines, R., Atta-Safah, S., Viray, E., Wondree, M.R., Klug, M.L., and Wagner, V.O., III, Evaluation of bacterial mutation historical control data, *Environ. Mol. Mutagen.*, 41, 169, 2003.
33. Kier, L.E., Brusick, D., Auletta, A.E., Von Halle, E.S., Brown, M.M., Simmon, V.F., Dunkel, V. et al., The *Salmonella typhimurium*/mammalian microsomal assay. A report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutat. Res.*, 168, 69, 1986.
34. Claxton, L.D., Allen, J., Auletta, A., Mortelmans, K., Nestmann, E., and Zeiger, E., Guide for the *Salmonella typhimurium*/mammalian microsome tests for bacterial mutagenicity, *Mutat. Res.*, 189, 83, 1987.
35. Clive, D., Caspary, W., Kirby, P.E., Krehl, R., Moore, M., Mayo, J., and Oberly, T.J., Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity, *Mutat. Res.*, 189, 143, 1987.
36. Li, A.P., Carver, J.H., Choy, W.N., Hsie, A.W., Gupta, R.S., Loveday, K.S., O'Neill, J.P., Riddle, J.C., Stankowski, L.F., Jr., and Yang, L.L., A guide for the performance of the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase gene mutation assay, *Mutat. Res.*, 189, 135, 1987.
37. OECD (Organisation of Economic Co-operation and Development), Test Guideline 476, OECD Guideline for testing of chemicals—*In vitro* mammalian cell gene mutation test, OECD, Paris, France, adopted July 21, 1997. http://www.oecd-ilibrary.org/environment/test-no-476-in-vitro-mammalian-cell-gene-mutation-test_9789264071322-en (accessed November 12, 2012).
38. DeMarini, D.M., Brockman, H.E., deSerres, F.J., Evans, H.E., Stankowski, L.F., Jr., and Hsie, A.W., Specific-locus mutations induced in eukaryotes (especially mammalian cells) by radiation and chemicals: A prospective, *Mutat. Res.*, 220, 11, 1989.
39. Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G., and Brown, M.M.M., Validation and characterization of the L5178Y TK⁺ mouse lymphoma mutagen assay system, *Mutat. Res.*, 59, 61, 1979.
40. Kozak, C.A. and Ruddle, F.H., Assignment of the genes for thymidine kinase and galactokinase to *Mus musculus* chromosome 11 and the preferential segregation of this chromosome with Chinese hamster/mouse somatic cell hybrids, *Somatic Cell Genet.*, 3, 121, 1977.
41. Hozier, J., Scalzi, J., Sawyer, J., Carley, N., Applegate, M., Clive, D., and Moore, M.M., Localization of the mouse thymidine kinase gene to the distal portion of chromosome 11, *Genomics*, 10, 827, 1991.
42. Moore, M.M., Clive, D., Howard, B.E., Batson, A.G., and Turner, N.T., *In situ* analysis of trifluorothymidine-resistant (TFT^r) mutants of L5178Y/TK⁺ mouse lymphoma cells, *Mutat. Res.*, 151, 147, 1985.
43. Mitchell, A.D., Auletta, A.E., Clive, D., Kirby, P.E., Moore, M.M., and Myhr, B.C., The L5178Y/tk⁺ mouse lymphoma specific gene and chromosomal mutation assay. A phase III report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutat. Res.*, 394, 177–303, 1997.
44. Moore, M.M., Honma, M., Clements, J., Harrington-Brock, K., Awogi, T., Bolcsfoldi, G., Cifone, M. et al., Mouse lymphoma thymidine kinase gene mutation assay: Follow-up International Workshop on Genotoxicity Test Procedures, New Orleans, Louisiana, April 2000, *Environ. Mol. Mutagen.*, 40(4), 292–299, 2002.
45. Moore, M.M., Honma, M., Clements, J., Bolcsfoldi, G., Burlinson, B., Cifone, M., Clarke, J. et al., Mouse lymphoma thymidine kinase gene mutation assay: Follow-up Meeting of the International Workshop on Genotoxicity Testing, Aberdeen, Scotland, 2003 Assay Acceptance Criteria, Positive Controls, and Data Evaluation, *Environ. Mol. Mutagen.*, 47, 1–5, 2006.
46. Moore, M.M., Honma, M., Clements, J., Bolcsfoldi, G., Burlinson, B., Cifone, M., Clarke, J. et al., Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005 recommendations for 24-hour treatment, *Mutat. Res.*, 627, 36–40, 2007.
47. Moore, M.M., Clive, D., Howard, B.E., Batson, A.G., and Turner, N.T., *In situ* analysis of trifluorothymidine-resistant (TFT^r) mutants of L5178Y/TK⁺ mouse lymphoma cells, *Mutat. Res.*, 151, 147, 1985.
48. Snee, R.D., and Irr, J.D., Design of a statistical method for the analysis of mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase locus of cultured Chinese hamster ovary cells, *Mutat. Res.*, 85, 77–83, 1981.
49. OECD (Organisation of Economic Co-operation and Development), Guideline for the testing of chemicals, Guideline 473, *In vitro* mammalian chromosome aberration test, Organization for Economic Cooperation and Development, Paris, adopted July 21, 1997. http://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test_9789264071261-en (accessed October 31, 2012).
50. OECD (Organisation of Economic Co-operation and Development), Guideline for the testing of chemicals, Proposal for Updating Guideline 473, *In vitro* mammalian chromosome aberration test, draft available for public comment by June 7, 2012. <http://www.oecd.org/env/chemicalsafetyandbiosafety/testingofchemicals/50108781.pdf> (accessed October 31, 2012).
51. Preston, R.J., Dean, B.J., Galloway, S., Holden, H., McFee, A.F., and Shelby, M., Mammalian *in vivo* cytogenetic assays. Analysis of chromosome aberrations in bone marrow cells, *Mutat. Res.*, 189, 157, 1987.

52. Swierenga, S.H.H., Heddle, J.A., Sigal, E.A., Gilman, J.P.W., Brillinger, R.L., Douglas, G.R., and Nestmann, E.R., Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese hamster lung and human lymphocyte cultures, *Mutat. Res.*, 246, 301, 1991.
53. Margolin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S.M., Bloom, A.D., and Zeiger, E., Statistical analyses for *in vitro* cytogenetic assays using Chinese hamster ovary cells, *Environ. Mutagen.*, 8, 183, 1986.
54. Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M., Jr., Brusick, D., Ashby, J., and Myhr, B.C., Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9, *Mutat. Res.*, 257, 147, 1991.
55. Hardman, J.G., Limbird, L.E., and Gilman, A.G., eds., Goodman & Gilman's, *The Pharmacological Basis of Therapeutics*, 10th edition. McGraw-Hill Professional, New York, August 13, 2001.
56. Morita, T., Watanabe, Y., Takeda, K., and Okumura, K., Effects of pH in the *in vitro* chromosomal aberration test, *Mutat. Res.*, 225, 55, 1989.
57. Galloway, S.M., Deasy, D.A., Bean, C.L., Kraynak, A.R., Armstrong, N.J., and Bradley, M.O., Effects of high osmotic strength on chromosome aberrations, sister-chromatid exchanges and DNA strand breaks, and the relation to toxicity, *Mutat. Res.*, 189, 15, 1987.
58. OECD (Organisation of Economic Co-operation and Development), Guideline for the testing of chemicals, Guideline 487, *In vitro* mammalian cell micronucleus test, Organization for Economic Cooperation and Development, Paris, adopted July 22, 2010. http://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264091016-en (accessed October 31, 2012).
59. Kirsch-Volders, M., Towards a validation of the micronucleus test, *Mutat. Res.*, 392, 1–4, 1997.
60. Parry, J.M. and Sors, A., The detection and assessment of the aneuploid potential of environmental chemicals: The European Community aneuploidy project, *Mutat. Res.*, 287, 3–15, 1993.
61. Fenech, M. and Morley, A.A., Solutions to the kinetic problem in the micronucleus assay, *Cytobios*, 43, 233–246, 1985.
62. Lorge, E., Hayashi, M., Albertini, S., and Kirkland, D., Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test. I. Theoretical aspects, *Mutat. Res.*, 655, 1–3, 2008.
63. Eastmond, D.A. and Tucker, J.D., Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody, *Environ. Mol. Mutagen.*, 13, 34–43, 1989.
64. Bryce, S.M., Bemis, J.C., Avlasevich, S.L., and Dertinger, S.D., *In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity, *Mutat. Res.*, 630, 78–91, 2007.
65. Shi, J., Bezabhe, R., and Szkudlinska, A., Further evaluation of a flow cytometric *in vitro* micronucleus assay in CHO-K1 cells: A reliable platform that detects micronuclei and discriminates apoptotic bodies, *Mutagenesis*, 25(1), 33–40, 2010.
66. OECD (Organisation of Economic Co-operation and Development), Guideline for the testing of chemicals, Guideline 475: Mammalian bone marrow chromosome aberration test, adopted July 21, 1997. http://www.oecd-ilibrary.org/environment/test-no-475-mammalian-bone-marrow-chromosome-aberration-test_9789264071308-en (accessed October 31, 2012).
67. OECD Guideline for the testing of chemicals, Proposal for Updating Guideline 475: Mammalian bone marrow chromosome aberration test, Draft available for public comment by July 7, 2012. <http://www.oecd.org/env/chemicalsafetyandbio-safety/testingofchemicals/Draft%20TG%20475.pdf> (accessed October 31, 2012).
68. Mitchell, A.D., Auletta, A.E., Clive, D., Kirby, P.E., Moore, M.M., and Myhr, B.C., The L5178Y/tk⁺ mouse lymphoma specific gene and chromosomal mutation assay. A phase III report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutat. Res.*, 394, 177–303, 1997.
69. Moore, M.M., Honma, M., Clements, J., Harrington-Brock, K., Awogi, T., Bolcsfoldi, G., Cifone, M. et al., Mouse lymphoma thymidine kinase gene mutation assay: Follow-up International Workshop on Genotoxicity Test Procedures, New Orleans, Louisiana, *Environ. Mol. Mutagen.*, 40(4), 292–299, 2002.
70. Kliesch, V., Danford, N., and Adler, I.-D., Micronucleus test and bone marrow chromosome analysis. A comparison of two methods *in vivo* for evaluating chemically induced chromosomal alterations, *Mutat. Res.*, 80, 321, 1981.
71. Heddle, J.A., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J.T., Newell, G.W., and Salamone, M.F., The induction of micronuclei as a measure of genotoxicity. A Report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutat. Res.*, 123, 61, 1983.
72. OECD (Organisation of Economic Co-operation and Development), Guideline for the testing of chemicals. Guideline 474, Mammalian erythrocyte micronucleus test, Organization for Economic Cooperation and Development, Paris, adopted August 1998. http://www.oecd-ilibrary.org/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264071285-en (accessed November 12).
73. MacGregor, J.T., Heddle, J.A., Hite, M., Morgolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R., and Wild, D., Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes, *Mutat. Res.*, 189, 103, 1987.
74. Hayashi, M., Sutou, S., Shimada, H., Sato, S., Sasaki, Y.F., and Wakata, A., Difference between intraperitoneal and oral gavage application in the micronucleus test. The 3rd collaborative study by CSGMT/JEMS-MMS, *Mutat. Res.*, 223, 329–344, 1989.
75. Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., and Wassom, J.S., Mammalian *in vivo* and *in vitro* cytogenetic assays: A report of the U.S. EPA's Gene-Tox Program, *Mutat. Res.*, 87, 143, 1981.
76. Wakata, A., Miyamae, Y., Sato, S., Suzuki, T., Morita, T., and Asano N., Evaluation of the rat micronucleus test with bone marrow and peripheral blood: Summary of the 9th collaborative study by CSGMT/JEMS. MMS, *Environ. Mol. Mutagen.*, 32, 84–100, 1998.
77. Hamada, S., Sutou, S., Morita, T., Wakata, A., Asanami, S., and Hosoya, S., Evaluation of the rodent micronucleus assay by a 28-day treatment protocol: Summary of the 13th collaborative study by the collaborative study group for the micronucleus test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)–Mammalian Mutagenicity Study, *Environ. Mol. Mutagen.*, 37, 93–110, 2001.
78. Hayashi, M., MacGregor, J.T., Gatehouse, D.G., Blakey, D.H., Dertinger, S.D., Abramsson-Zetterberg, L., Krishna, G. et al., *In vivo* erythrocyte micronucleus assay. III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test, *Mutat. Res.*, 627, 10–30, 2007.

79. MacGregor, J.T., Bishop, M.E., McNamee, J.P., Hayashi, M., Asano, N., Wakata, A., Nakajima, M., Saito, J., Aidoo, A., Moore, M.M., and Dertinger, S.D., Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: II. An efficient method of monitoring chromosomal damage in the rat, *Toxicol. Sci.*, 94, 92–107, 2006.
80. Kissling, G.E., Dertinger, S.D., Hayashi, M., and MacGregor, J.T., Sensitivity of the erythrocyte micronucleus assay: Dependence on number of cells scored and inter-animal variability, *Mutat. Res.*, 634, 235–240, 2007.
81. Hayashi, M., MacGregor, J.T., Gatehouse, D.G., Adler, I., Blakey, D.H., Dertinger, S.D., Krishna, G., Morita, T., Russo, A., and Sutou, S., *In vivo* rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring, *Environ. Mol. Mutagen.*, 35, 234–252, 2000.
82. Torous, D.K., Hall, N.E., Illi-Love, A.H., Diehl, M.S., Cederbrant, K., Sandelin, K., Ponten, I. et al., Interlaboratory validation of a CD71-based flow cytometric method (Microflow) for the scoring of micronucleated reticulocytes in mouse peripheral blood, *Environ. Mol. Mutagen.*, 45(1), 44–55, 2005.
83. Elhajouji, A., Lukamowicz, M., Cammerer, Z., and Kirch-Volders, M., Potential threshold for genotoxic effects by micronucleus scoring, *Mutagenesis*, 26, 199, 2011.
84. Kirsch-Volders, M., Elhajouji, A., Cundari, E., and Hummelen P.V., The *in vitro* micronucleus test: A multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction, *Mutat. Res.*, 392, 19, 1997.
85. Norppa, H. and Falck, G.C.-M., What do human micronuclei contain? *Mutagenesis*, 18, 221, 2003.
86. Eastmond, D.A. and Pinkel, D., Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence *in situ* hybridization with chromosome-specific DNA probes, *Mutat. Res.*, 234, 9, 1990.
87. Miller, B.M. and Adler, I.D., Application of antikinetochore antibody staining (CREST staining) to micronuclei in polychromatic erythrocytes induced *in vivo*, *Mutagenesis*, 5, 411, 1990.
88. Schuler, M., Rupa, D.S., and Eastmond, D.A., A critical evaluation of centromeric labeling to distinguish micronuclei induced by chromosomal loss and breakage *in vitro*, *Mutat. Res.*, 392, 81, 1997.
89. Fenech, M., The *in vitro* micronucleus technique, *Mutat. Res.*, 455, 81, 2000.
90. Abend, A., Blakely, W.F., and van Beuningen, D., Simplified and optimized kinetochore detection: Cytogenetic marker for late-G2 cells, *Mutat. Res.*, 334, 39, 1995.
91. FDA (US Food and Drug Administration), Revised July 2000, Redbook 2000, Guidance for industry and other stakeholders toxicological principles for the safety assessment of food ingredients—Redbook 2000. US FDA, Center for Food Safety and Applied Nutrition. <http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm> (accessed November 2, 2012).
92. Östling, O. and Johanson, K.J., Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells, *Biochem. Biophys. Res. Commun.*, 123, 291–298, 1984.
93. Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider E.L., A simple technique for quantification of low levels of DNA damage in individual cells, *Exp. Cell Res.*, 175, 184–191, 1988.
94. Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., and Sasaki, Y.F., Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing, *Environ. Mol. Mutagen.*, 35, 206–221, 2000.
95. Sasaki, Y.F., Kawaguchi, S., Kamaya, A., Ohshita, M., Kabasawa, K., Iwama, K., Taniguchi, K., and Tsuda, S., The comet assay with 8 mouse organs: Results with 39 currently used food additive, *Mutat. Res.*, 519, 103–119, 2002.
96. Sekihashi, K., Yamamoto, A., Matsumura, Y., Ueno, S., Watanabe-Akanuma, M., Kassie, F., Knasmüller, S., Tsuda, S., and Sasaki, Y.F., Comparative investigation of multiple organs of mice and rats in the comet assay, *Mutat. Res.*, 517, 53–75, 2002.
97. Hartmann, A., Agurell, D., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V., and Tice, R.R., Recommendations for conducting the *in vivo* alkaline comet assay, *Mutagenesis*, 18(1), 45–51, 2003.
98. Sekihashi, K., Yamamoto, A., Matsumura, Y., Ueno, S., Watanabe-Akanuma, M., Kassie, F., Knasmüller, S., Tsuda, S., and Sasaki, Y.F., Comparative investigation of multiple organs of mice and rats in the comet assay, *Mutat. Res.*, 517, 53–75, 2002.
99. Burlinson, B., Tice, R.R., Speit, G., Agurell, E., Brendler-Schwaab, S., Collins, A.R., Escobar, P. et al., Fourth International Workgroup on Genotoxicity Testing: Result of the *in vivo* comet assay work, *Mutat. Res.*, 627, 31–35, 2007.
100. JaCVAM, The Validation Management Team (VMT) coordinated by JaCVAM (Japanese Committee for the Validation of Alternative Methods), International validation of the *in vivo* rodent alkaline comet assay for the detection of genotoxic carcinogens (Protocol Version 14.2), November 30, 2009. <http://cometassay.com/JaCVAM.pdf> (accessed October 30, 2012).
101. Olive, P.L., Banath, J.P., and Durand, R.E., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the “comet” assay, *Radiat. Res.*, 122(1), 86–94, 1990.
102. Collins, A.R., The comet assay for DNA damage and repair: Principles, applications, and limitations, *Mol. Biotechnol.*, 26, 249–261, 2004.
103. Lovell, D.P., Thomas, G., and Dubow, R., Issues related to the experimental design and subsequent statistical analysis of *in vivo* and *in vitro* comet studies, *Teratogen. Carcinogen. Mutagen.*, 19(2), 109–119, 1999.
104. Wiklund, S.J. and Agurell, E., Aspects of design and statistical analysis in the comet assay, *Mutagenesis*, 18(2), 167–175, 2003.
105. OECD (Organisation of Economic Co-operation and Development), Guideline for Testing of Chemicals. 2011. Test Guideline 488—Transgenic rodent somatic and germ cell gene mutation assays, adopted July 28, 2011, OECD, Paris, France. http://www.oecd-ilibrary.org/environment/test-no-488-transgenic-rodent-somatic-and-germ-cell-gene-mutation-assays_9789264122819-en (accessed November 2, 2012).
106. Lambert, I.B., Singer, T.M., Boucher, S.E., and Douglas, G.R., Detailed review of transgenic rodent mutation assays, *Mutat. Res.*, 590(1–3), 1–280, 2005.
107. OECD (Organisation of Economic Co-operation and Development), Detailed review paper on transgenic rodent mutation assays, Series on Testing and Assessment, No. 103, ENV/JM/MONO(2009)7, OECD, Paris, France, 2009. <http://www.oecd.org/chemicalsafety/testingofchemicals/seriesontestingandassessmentpublicationsbynumber.htm> (accessed November 2, 2012).

108. OECD (Organisation of Economic Co-operation and Development), Transgenic rodent somatic and germ cell gene mutation assays: Retrospective performance assessment, Series on Testing and Assessment, Number 145, ENV/JM/MONO 20, OECD, Paris, France, 2011. <http://www.oecd.org/chemicalsafety/testingofchemicals/48532170.pdf> (accessed November 2, 2012).
109. Heddle, J.A., Dean, S., Nohmi, T., Boerrigter, M., Casciano, D., Douglas, G.R., Glickman, B.W. et al., *In vivo* transgenic mutation assays, *Environ. Mol. Mutagen.*, 35, 253–259, 2000.
110. Thybaud, V., Dean, S., Nohmi, T., de Boer, J., Douglas, G.R., Glickman, B.W., Gorelick N.J., Heddle J.A., Heflich, R.H., Lambert, I., Martus, H.-J., Mirsalis, J.C., Suzuki, T., and Yajima, N., *In vivo* transgenic mutation assays, *Mutat. Res.*, 540, 141–151, 2003.
111. Müller, L., Gocke, E., Lavé, T., and Pfister, T., Ethyl methane-sulfonate toxicity in Viracept—A comprehensive human risk assessment based on threshold data for genotoxicity, *Toxicol. Lett.*, 190(3), 317–329, 2009.
112. Dean, S.W., Brooks, T.M., Burlinson, B., Mirsalis, J., Myhr, B., Recio, L., and Thybaud, V., Transgenic mouse mutation assay systems can play an important role in regulatory mutagenicity testing *in vivo* for the detection of site-of-contact mutagens, *Mutagenesis*, 14(1), 141–151, 1999.
113. Gossen, J.A., de Leeuw, W.J., Verwest, A., Lohman, P.H., and Vijg, J., High somatic mutation frequencies in a *lacZ* transgene integrated on the mouse X-chromosome, *Mutat. Res.*, 250(1–2), 423–429, 1991.
114. Vijg, J. and Douglas, G.R., Bacteriophage lambda and plasmid *lacZ* transgenic mice for studying mutations *in vivo*, in *Technologies for Detection of DNA Damage and Mutations, Part II*, Pfeifer, G., ed. Plenum Press, New York, pp. 391–410, 1996.
115. Kohler, S.W., Kretz, P.L., Fieck, A., Sorge, J.A., and Short, J.M., The use of transgenic mice for short-term, *in vivo* mutagenicity testing, *Genet. Anal. Tech. Appl.*, 7(8), 212–218, 1990.
116. Provost, G.S., Kretz, P.L., Hamner, R.T., Matthews, C.D., Rodgers, B.J., Lundberg, K.S., Dyaico, M.J., and Short, J.M., Transgenic systems for *in vivo* mutation analysis, *Mutat. Res.*, 288, 133, 1993.
117. Kohler, S.W., Provost, G.S., Fieck, A., Kreta, P.L., Bullock, W.O., Sorge, J.A., Putman, D.L., and Short, J.M., Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 7958, 1991.
118. Jakubczak, J.L., Merlino, G., French, J.E., Muller, W.J., Paul, B., Adhya, S., and Garges, S., Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for *in vivo* mutations in a bacteriophage lambda transgene target, *Proc. Natl. Acad. Sci. U.S.A.*, 93(17), 9073–9078, 1996.
119. Watanabe, R., Kinoshita, T., Masaki, R., Yamamoto, A., Takeda, J., and Inoue, N., PIG-A and PIG-H, which participate in glycosylphosphatidylinositol anchor biosynthesis, form a protein complex in the endoplasmic reticulum, *J. Biol. Chem.*, 271, 26868–26875, 1996.
120. Miura, D., Dobrovolsky, V.N., Mittelstaedt, R.A., Kasahara, Y., Katsuura, Y., and Heflich, R.H., Development of an *in vivo* gene mutation assay using the endogenous *Pig-A* gene: II. Selection of *Pig-A* mutant rat spleen T-cells with proaerolysin and sequencing *Pig-A* cDNA from the mutants, *Environ. Mol. Mutagen.*, 49, 622–630, 2008.
121. Kimoto, T., Suzuki, K., Kobayashi, X.M., Dobrovolsky, V.N., Heflich, R.H., Miura, D., and Kasahara, Y., Manifestation of *Pig-a* mutant bone marrow erythroids and peripheral blood erythrocytes in mice treated with *N*-ethyl-*N*-nitrosourea: Direct sequencing of *Pig-a* cDNA from bone marrow cells negative for GPI-anchored protein expression, *Mutat. Res.*, 723, 36–42, 2011.
122. Araten, D.J., Nafa, K., Pakdeesuwan, K., and Luzzatto, L., Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals, *Proc. Natl. Acad. Sci. U.S.A.*, 96, 5209–5214, 1999.
123. Bryce, S.M., Bemis, J.C., and Dertinger, S.D., *In vivo* mutation assay based on the endogenous *Pig-a* locus, *Environ. Mol. Mutagen.*, 49, 256–264, 2008.
124. Miura, D., Dobrovolsky, V.N., Mittelstaedt, R.A., Kasahara, Y., Katsuura, Y., and Heflich, R.H., Development of an *in vivo* gene mutation assay using the endogenous *Pig-A* gene: II. Selection of *Pig-A* mutant rat spleen T-cells with proaerolysin and sequencing *Pig-A* cDNA from the mutants, *Environ. Mol. Mutagen.*, 49, 622–630, 2008.
125. Miura, D., Dobrovolsky, V.N., Kasahara, Y., Katsuura, Y., and Heflich, R.H., Development of an *in vivo* gene mutation assay using the endogenous *Pig-A* gene: I. Flow cytometric detection of CD59-negative peripheral red blood cells and CD48-negative spleen T cells from the rat, *Environ. Mol. Mutagen.*, 49, 614–621, 2008.
126. Phonethepswath, S., Bryce, S.M., Bemis, J.C., and Dertinger, S.D., Erythrocyte-based *Pig-a* gene mutation assay: Demonstration of cross-species potential, *Mutat. Res.*, 657, 122–126, 2008.
127. Dertinger, S.D., Phonethepswath, S., Weller, P., Nicolette, J., Murray, J., Sonders, P., Vohr, H.-W. et al., International *Pig-a* gene mutation assay trial: Evaluation of transferability across fourteen laboratories, *Environ. Mol. Mutagen.*, 52, 690–698, 2011.
128. Phonethepswath, S., Franklin, D., Torous, D.K., Bryce, S.M., Bemis, J.C., Raja, S., Avlasevich, S. et al., *Pig-a* mutation: Kinetics in rat erythrocytes following exposure to five prototypical mutagens, *Toxicol. Sci.*, 114, 59–70, 2011.
129. Bhalli, J.A., Shaddock, J.G., Pearce, M.G., Dobrovolsky, V.N., Cao, X., Heflich, R.H., and Vohr, H.-W., Report on Stage III *Pig-a* mutation assays using benzo[*a*]pyrene, *Environ. Mol. Mutagen.*, 52, 731–737, 2011b.
130. Lynch, A.M., Giddings, A., Custer, L., Gleason, C., Henwood, A., Aylott, M., and Kenny, J., International *Pig-a* gene mutation assay trial (Stage 3): Results with *N*-methyl-*N*-nitrosourea, *Environ. Mol. Mutagen.*, 52, 699–710, 2011.
131. Shi, J., Krsmanovic, L., Bruce, S., Kelly, T., Paranjpe, M., Szabo, K., Arevalo, M. et al., Assessment of genotoxicity induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) or diethylnitrosamine (DEN) in the *Pig a*, micronucleus and comet assays integrated into 28-day repeat dose studies, *Environ. Mol. Mutagen.*, 52, 711–720, 2011.
132. Stankowski, L.F., Jr., Roberts, D.J., Chen, H., Lawlor, T., McKeon, M., Murli, H., Thakur, A., and Xu, Y., Integration of *Pig-a*, micronucleus, chromosome aberration, and comet assay endpoints in a 28-day rodent toxicity study with 4-nitroquinoline-1-oxide (4NQO), *Environ. Mol. Mutagen.*, 52, 738–747, 2011.
133. Cammerer, Z., Bhalli, J.A., Cao, X., Coffing, S.L., Dickinson, D., Dobo, K.L., Dobrovolsky, V.N. et al., Report on Stage III *Pig-a* mutation assays using *N*-ethyl-*N*-nitrosourea—Comparison with other *in vivo* genotoxicity endpoints, *Environ. Mol. Mutagen.*, 52, 721–730, 2011.

134. Bhalli, J.A., Pearce, M.G., Dobrovolsky, V.N., and Heflich, R.H., Manifestation and persistence of Pig-a mutant red blood cells in C57BL/6 mice following single and split doses of *N*-ethyl-*N*-nitrosourea, *Environ. Mol. Mutagen.*, 52, 766–773, 2011.
135. Lemieux, C.L., Douglas, G.R., Gingerich, J., Phonetheswath, S., Torous, D.K., Dertinger, S.D., Phillips, D.H., Arlt, V.M., and White, P.A., Simultaneous measurement of benzo[*a*]pyrene-induced Pig-a and *lacZ* mutations, micronuclei and DNA adducts in Muta™ mouse, *Environ. Mol. Mutagen.*, 52, 756–765, 2011.
136. Dobrovolsky, V.N., Elespuru, R.K., Bigger, C.A.H., Robison, T.W., and Heflich, R.H., Monitoring humans for somatic mutation in the endogenous *Pig-a* gene using red blood cells, *Environ. Mol. Mutagen.*, 52, 784–794, 2011.
137. Dobrovolsky, V.N., Shaddock, J.G., Mittelstaedt, R.A., Manjanatha, M.G., Miura, D., Uchikawa, M., Mattison, D.R., and Morris, S.M., Evaluation of *Macaca mulatta* as a model for genotoxicity studies, *Mutat. Res.*, 673, 21–28, 2009.
138. Ohtani, S., Unno, A., Ushiyama, A., Kimoto, T., Miura, D., and Kunugita, N., The *in vivo* *Pig-a* gene mutation assay is useful for evaluating the genotoxicity of ionizing radiation in mice, *Environ. Mol. Mutagen.*, 53, 579–588, 2012.
139. Dertinger, S.D., Bryce, S., Phonetheswath, S., and Avlasevich, S., When pigs fly: Immunomagnetic separation facilitates rapid determination of Pig-a mutant frequency by flow cytometric analysis, *Mutat. Res.*, 721, 163–170, 2011.
140. Dobo, K.L., Fiedler, R.D., Gunther, W.C., Thiffeault, C.J., Cammerer, Z., Coffing, S.L., Shutsky, T., and Schuler, M., Defining EMS and ENU dose–response relationships using the Pig-a mutation assay in rats, *Mutat. Res.*, 725, 13–21, 2011.
141. Ohtani, S., Unno, A., Ushiyama, A., Kimoto, T., Miura, D., and Kunugita, N., The *in vivo* *Pig-a* gene mutation assay is useful for evaluating the genotoxicity of ionizing radiation in mice, *Environ. Mol. Mutagen.*, 53, 579–588, 2012.
142. Michalopoulos, G., Sattler, C.A., Sattler, G.L., and Pitot, H.C., Cytochrome P-450 induction by phenobarbital and 3-methylcholanthrene in primary cultures of hepatocytes, *Science*, 193, 907, 1976.
143. Mirsalis, J.C., Tyson, K.C., and Butterworth, B.E., The detection of genotoxic carcinogens in the *in vivo*–*in vitro* hepatocyte DNA repair assay, *Environ. Mutagen.*, 4, 553, 1982.
144. Butterworth, B.E., Ashby, J., Bermudez, E., Casciano, D., Mirsalis, J., Probst, G., and Williams, G., A protocol and guide for the *in vivo* rat hepatocyte DNA-repair assay, *Mutat. Res.*, 189, 123, 1987.
145. San, R.H.C., Sly, J.E., and Raabe, H.A., Unscheduled DNA synthesis in rat hepatocytes following *in vivo* administration of dimethylnitrosamine via different routes, *Environ. Mol. Mutagen.*, 27(Suppl 27), 58, 1996.
146. Mirsalis, J.C. and Butterworth, B.E., Detection of unscheduled DNA synthesis in hepatocytes isolated from rats treated with genotoxic agents: An *in vivo*–*in vitro* assay for potential carcinogens and mutagens, *Carcinogenesis*, 1, 621, 1980.
147. Ashby, J., Lefevre, P.A., Burlinson, B., and Penman, M.G., An assessment of the *in vivo* hepatocyte DNA-repair assay, *Mutat. Res.*, 156, 1, 1985.
148. Barrett J.C. and Ts'o, P.O.P., Evidence for the progressive nature of neoplastic transformation *in vitro*, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3761–3765, 1978.
149. Kakunaga, T.H. and Kamasaki, H., eds., *Transformation Assay of Established Cell Lines: Mechanisms and Application*. IARC Scientific Publications No. 67. International Agency for Research on Cancer, Lyon, 225 p. 1985.
150. Berwald, Y. and Sachs, L., *In vitro* cell transformation with chemical carcinogens, *Nature*, 200, 1182–1184, 1963.
151. Newbold, R.F., Overell, R.W., and Connell, J.R., Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens, *Nature*, 299, 633–635, 1982.
152. Elias, Z., Poirot, O., Pezerat, H., Suquet, H., and Schneider, O., Cytotoxic and neoplastic effects of industrial hexavalent chromium pigments in Syrian hamster embryo cells, *Carcinogenesis*, 10(11), 2043–2052, 1989.
153. Creton, S., Aardema, M.J., Carmichael, P.L., Harvey, J.S., Martin, F.L., Newbold, R.F., O'Donovan, M.R. et al., Cell transformation assays for prediction of carcinogenic potential: State of the science and future research needs, *Mutagenesis*, 27, 93–101, 2012.
154. Vanparys, P., Corvi, R., Aardema, M., Gribaldo, L., Hayashi, M., Hoffmann, S., and Schechtman, L., ECVAM prevalidation of three cell transformation assays, *ALTEX*, 28(1), 56–59, 2011.
155. Sasaki, K., Mizusawa, H., and Ishidate, M., Isolation and characterization of ras-transfected BALB/3T3 clone showing morphological transformation by 12-*O*-tetradecanoylphorbol-13-acetate, *Jpn. J. Cancer Res.*, 79, 921–930, 1988.
156. Sasaki, K., Mizusawa, H., Ishidate, M., and Tanaka, N., Establishment of a highly reproducible transformation assay of a ras-transfected BALB/3T3 clone by treatment with promoters, *Basic Life Sci.*, 52, 411–416, 1990.
157. Asada, S., Sasaki, K., Tanaka, N., Takeda, K., Hayashi, M., and Umeda, M., Detection of initiating activities of chemicals using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells), *Mutat. Res.*, 588, 7–21, 2005.
158. Kerckaert, G.A., Isfort, R.J., Carr, G.J., Aardema, M.J., and LeBoeuf, R.A., A comprehensive protocol for conducting the Syrian hamster embryo cell transformation assay at pH 6.70, *Mutat. Res.*, 356, 65–84, 1996.
159. Pant, K., Bruce, S.W., Sly, J.E., Kunkelmann, T., Bohnenberger, S., Poth, A., Engelhardt, G., Schulz, M., and Schwind, K.-R., Prevalidation study of the Syrian hamster embryo (SHE) cell transformation assay at pH 6.7 for assessment of carcinogenic potential of chemicals, *Mutat. Res.*, 744, 54–63, 2012.
160. Maire, M.-A., Pant, K., Phrakonkham, P., Poth, A., Schwind, K.-R., Rast, C., Bruce, S.W. et al., Recommended protocol for the Syrian hamster embryo (SHE) cell transformation assay, *Mutat. Res.*, 744, 76–81, 2012.
161. Maire, M.-A., Pant, K., Poth, A., Schwind, K.-R., Rast, C., Bruce, S.W., Sly, J.E. et al., Prevalidation study of the Syrian hamster embryo (SHE) cell transformation assay at pH 7.0 for assessment of carcinogenic potential of chemicals, *Mutat. Res.*, 744, 64–75, 2012.
162. Isfort, R.J., Cody, D.B., Doerson, C., Kerckaert, G.A., and LeBoeuf, R.A., Alterations in cellular differentiation, mitogenesis, cytoskeleton and growth characteristics during Syrian hamster embryo cell multistep *in vitro* transformation, *Int. J. Cancer*, 59, 114–125, 1994.
163. Bohnenberger, S., Kunkelmann, T., Perschbcher, S., Poth, A., Pant, K., Bruce, S.W., Sly, J.E., and Schwind, K.-R., Photo catalogue for the classification of cell colonies in the Syrian hamster embryo (SHE) cell transformation assay at pH 6.7, *Mutat. Res.*, 744, 82–96, 2012.
164. Gee, P., Sommers, C.H., Melick, A.S., Gidrol, X.M., Todd, M.D., Burris, R.B., Nelson, M.E., Klemm, R.C., and Zeiger, E., Comparison of responses of base-specific *Salmonella*

- tester strains with the traditional strains for identifying mutagens: The results of a validation study, *Mutat. Res.*, 412, 115–130, 1998.
165. ISO 10993-1, International Organization for Standardization, Biological evaluation of medical devices Part 1: Evaluation and testing in the risk management process, Geneva, Switzerland, 2009. http://www.iso.org/iso/catalogue_detail.htm?csnumber=44908 (accessed November 12, 2012).
166. ISO 10993-3, International Organization for Standardization, Biological evaluation of medical devices Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity, Geneva, Switzerland, 2003. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=32162 (accessed November 12, 2012).
167. ICH (International Conference on Harmonisation), ICH Test Guideline S6: Preclinical safety evaluation of biotechnology-derived pharmaceuticals, ICH Step 4, July 16, 1997. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S6_R1/Step4/S6_R1_Guideline.pdf (accessed November 12, 2012).
168. US EPA, Health Effects Test Guidelines: OPPTS 870.5100 Bacterial reverse mutation test, Washington, DC, 1998. <http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2009-0156-0022> (accessed November 12, 2012).
169. US EPA, Health Effects Test Guidelines: OPPTS 870.5395 Mammalian erythrocyte micronucleus test, Washington, DC, 1998. <http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2009-0156-0032> (accessed November 12, 2012).
170. Auletta, A., Dearfield, K.L., and Cimino, M.C., Mutagenicity test schemes and guidelines: U.S. EPA Office of Pollution Prevention and Toxics and Office of Pesticide Programs, *Environ. Mutagen.*, 21, 38, 1993.
171. US EPA, Pesticides and Science Policy, Advances in genetic toxicology and integration of *in vivo* testing into standard repeat dose studies, 2012. <http://www.epa.gov/pesticides/science/integrating-gentox-studies.html> (accessed November 12, 2012).
172. MHLW (Ministry of Health, Labour, and Welfare), Welcome page, Tokyo, Japan, 2012. <http://www.mhlw.go.jp/english/> (accessed November 13, 2012).
173. Sofuni, T., Japanese guidelines for mutagenicity testing, *Environ. Mutagen.*, 21, 2, 1993.
174. MAFF (Ministry of Agriculture, Forestry and Fisheries), Welcome page, Tokyo, Japan, 2012. <http://www.maff.go.jp/e/index.html> (accessed November 13, 2012).
175. METI (Ministry of Economy, Trade and Industry), Welcome page, Tokyo, Japan, 2012. <http://www.meti.go.jp/english/> (accessed November 13, 2012).
176. CPMP, Commission of the European Communities: The Rules Governing Medicinal Products in the European Community, Vol. III, Guidelines on the Quality, Safety and Efficacy of Medicinal Products for Human Use, p. 103, 1989.
177. REACH, Regulation (EC) No. 1907/2006 of the European Parliament and the Council Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), 2006. <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32006R1907:EN:NOT> (accessed November 13, 2012).

14 Carcinogenesis

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INTRODUCTION

Cancer ranks as the toxic effect of most concern to the public. Because of this, considerable effort and financial resources are spent annually to identify potential human carcinogens. The purpose of this chapter is to supply basic information pertaining to carcinogenesis in rodents which is essential for understanding and interpreting the results of chronic rodent carcinogenicity studies.

This chapter is divided into four sections:

1. An overview of mechanisms hypothesized to be involved in chemical carcinogenesis
2. Data on spontaneous carcinogenesis in several strains of mice and rats commonly used in chronic bioassays
3. Information pertaining to the design of the chronic rodent carcinogenicity study and the susceptibility of various rodent organs/tissues to chemically induced carcinogenesis
4. Basic tumor pathology

Information pertaining to cancer in humans can be found in Chapter 22 on Risk Assessment.

MECHANISMS OF CARCINOGENESIS

It is currently believed that the development of malignant tumors does not result from a single event but from a multistep process consisting of discrete but interrelated biological events. Evidence for a multistage process of carcinogenesis exists for a variety of animal organ systems including skin, liver, urinary bladder, lung, kidney, intestines, and pancreas.¹

In the multistage model of carcinogenesis, development of a single cell into a malignant tumor occurs in three stages: initiation, promotion, and progression. Initiation involves an irreversible change in a normal cell (usually an alteration

of the genome) allowing for unrestricted growth. The initiated cell may remain latent for months or years. During this period of latency, the initiated cell is phenotypically indistinguishable from surrounding cells. Further development of the initiated cell into a neoplastic cell requires a period of promotion. Under the influence of a promoter, tumor formation is accelerated through clonal expansion of initiated cells. Promoters, which do not directly interact with DNA, are a diverse group of agents believed to act via a variety of proposed mechanisms most often resulting in increased cell proliferation. The process of promotion is considered reversible and requires prolonged and repeated exposure to promoter agents.

Progression is the final step in which preneoplastic foci develop into malignant cells. In this stage, tumor development is characterized by karyotypic changes, increased growth rate, and invasiveness. The reader is referred to the following review articles for a more detailed discussion of multistage carcinogenesis: Pitot and Dragon,² Maronpot,¹ Pitot,³ and Butterworth and Goldsworthy.⁴

This section presents basic characteristics of initiator, promoter, and progressor agents, provides a brief overview of the multistage carcinogenesis model, and offers a classification of carcinogenic chemicals according to proposed mode of action (Figures 14.1 and 14.2 and Tables 14.1 through 14.3).

Figure 14.1 shows a model of the process of multistage carcinogenesis consisting of three stages: initiation, promotion, and progression. In this model, an initiator irreversibly alters genetic information of a normal cell. With prolonged exposure to a promoter, clonal expansion of the initiated cell occurs, usually the result of enhanced cellular proliferation, “fixing” the genetic change caused by the initiator and resulting in the formation of preneoplastic foci. This step can be reversible with insufficient exposure to the promoter. The critical step in carcinogenic process involves progression of the preneoplastic cells to malignancy. Under the influence of progressor agents, karyotypic changes

occur accompanied by increased growth rate and invasiveness leading to metastasis. This process is considered irreversible. During progression, both benign and malignant tumors are usually observed. Controversy exists as to whether a “true” benign tumor can progress to malignancy or whether the “Benign” tumor that progresses to malignancy contains malignant cells localized *in situ* which have not yet invaded beyond the basement membrane of the developing tumor.

Figure 14.2 is a schematic of a typical study designed to assess initiating and promoting potential of chemicals. To simulate cell proliferation, a partial hepatectomy (~70% of the liver) is performed on four groups of rats on the first day of the study (day 0). To investigate initiating activity, the suspect chemical in vehicle or vehicle alone is administered 24 h after hepatectomy (day 1). The single administration of the suspect chemical is generally by the oral route although other exposure routes are used. Beginning on day 7 of the

study and continuing for 8 weeks, a known promoter is administered to the animals (usually sodium phenobarbital, 500 ppm) in drinking water or water alone. At the end of 8 weeks, the animals are sacrificed and livers examined microscopically for the presence of foci of altered hepatocytes which have been selectively stained for γ -glutamyl transpeptidase as a marker. The response is quantitated as the number of foci per area of tissue. An increase in the number of foci only in the group treated with the promoter indicates that the suspect chemical is a “pure” initiator. An increase in foci in rats both with and without promoter treatment indicates that the suspect chemical possesses both initiating and promoting activity. Using the same study design, a chemical can be assessed for promoting potential by giving a known initiator on day 1 of the study (usually diethylnitrosamine, 50 mg/kg PO) and administering the suspect chemical in drinking water during the 8 week promotional period.

TABLE 14.1
Characteristics of Initiation, Promotion, and Progression

Initiation	Promotion	Progression
Irreversible	Reversible	Irreversible
Additive	Nonadditive	Karyotypic abnormalities appear accompanied by increase growth rate and invasiveness
Dose response can be demonstrated; does not exhibit a readily measurable threshold	Dose response having a measurable threshold can be demonstrated	Benign and/or malignant tumor observed
No measurable maximum response	Measurable maximum effect	Environmental factors influence early stage of progression
Initiators are usually genotoxic	Promoters are usually not mutagenic	Progressors may not be initiators
One exposure may be sufficient	Prolonged and repeated exposure to promoters required	Progressors act to advance promoted cells to a potentially malignant stage
Must occur prior to promotion	Promoter effective only after initiation has occurred	Spontaneous progression can occur
Requires fixation through cell division	Promoted cell population dependent on continued presence of promoter	
Initiated cells are not identifiable except as foci lesions following a period of promotion	Causes expansion of the progeny of initiated cells producing foci lesions	
“Pure” initiation does not result in neoplasia without promotion	“Pure” promoters not capable of initiation	
Spontaneous (fortuitous) initiation can occur	Sensitive to hormonal and dietary factors	

Sources: Adapted from Pitot, H.C., *Proc. Soc. Exp. Biol. Med.*, 198, 661, 1991; Maronpot, R.R., *Handbook of Toxicologic Pathology*, Haschek, W.M. and Rousseaux, C.G., Eds., Academic Press, San Diego, CA, 1991, Chapter 7.

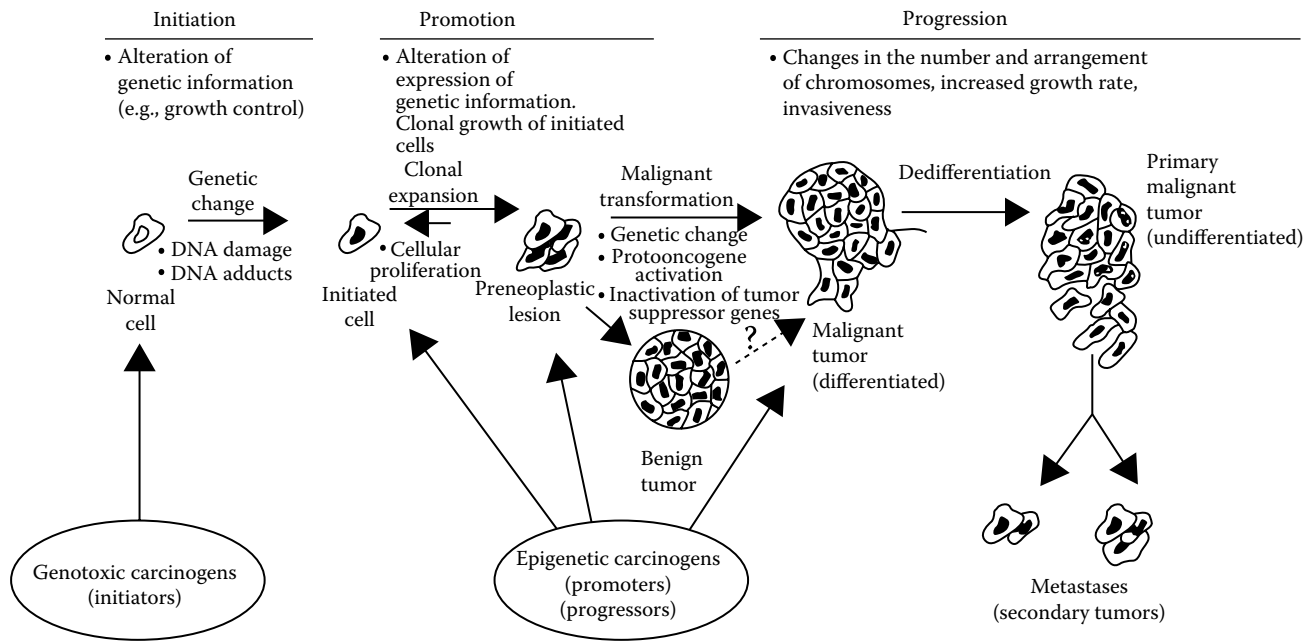


FIGURE 14.1 Multistage carcinogenesis. (Adapted from Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 6.)

TABLE 14.2
Defense Mechanisms and Self-Limiting Processes That Could Prevent Initiation or Progression

- Mutagens may be metabolized to nonmutagenic metabolites
- Genotoxic carcinogens may bind to materials other than DNA such as proteins or glutathione
- Error-free DNA repair processes may repair damage to DNA before the damage is fixed by DNA replication
- Damage to DNA may occur at sites not involved in carcinogenesis
- Damage to DNA may result in cell death before cell replication
- Initiated cells may be destroyed by the immune system
- Other critical events in multistage carcinogenesis do not occur or do not occur at critical times

Source: Adapted from Maynard, R.L. et al., *Human Exp. Toxicol.*, 14, 175, 1995.

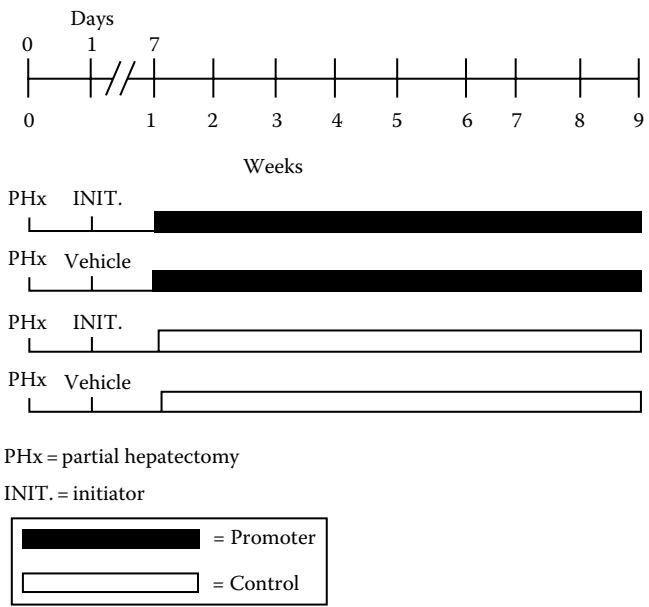


FIGURE 14.2 Initiation/promotion study design. (Adapted from Yager, J.P. et al., *Proc. Soc. Exp. Biol. Med.*, 198, 667, 1991.)

TABLE 14.3
Classification of Carcinogenic Chemicals Based on Mode of Action

Classification ^a	Mode of Action	Examples
I. Genotoxic	Agents which interact with DNA	
1. Direct acting (primary carcinogen; activation-independent)	Organic chemicals; direct alteration of DNA, chromosome structure, or number; metabolic conversion not required; generation of reactive electrophiles and covalent binding to DNA	Bis-chloromethylether, β -propiolactone, ethylene imine
2. Procarcinogen (secondary carcinogen; activation-dependent)	Organic chemicals; requires biotransformation to a direct acting carcinogen (proximate carcinogen)	Nitrosamines, ethylene dibromide, vinyl chloride
3. Inorganic carcinogen	Direct effects on DNA may occur through interference with DNA replication	Nickel, cadmium
II. Epigenetic	Agents for which there is no direct evidence of interaction with DNA	
4. Cytotoxin	Cytotoxic; induction of regenerative cell proliferation; mutations may occur secondarily through several mechanisms including release of nucleases, generation of reactive oxygen radicals, DNA replication before adduct repair; preferential growth of preneoplastic cells may be caused by selective killing of normal cells or expression of growth control genes (oncogenes)	Nitrilo triacetic acid, chloroform
5. Mitogen	Stimulation of mitogenic cell proliferation directly or via a cellular receptor; mutations may occur secondarily as a result of increased cell proliferation; preferential growth of preneoplastic cells may be caused through alteration of rates of cell birth or death	Phenobarbital, α -hexachlorocyclohexane
6. Peroxisome proliferator	Generation of reactive oxygen radicals through perturbation of lipid metabolism; growth control genes may be activated directly or via a cellular receptor	Fenofibrate, diethylhexyl phthalate, clofibrate
7. Immunosuppressor	Enhancement of the development of virally induced, transplanted, and metastatic neoplasms possibly through impairment or loss of natural and acquired tumor resistance	Azathioprine, cyclosporin A, 6-mercaptopurine
8. Hormones and hormonal altering agents	Chronic stimulation of cell growth through activation of regulatory genes; other potential modes of action include promotional effects resulting from alteration of hormonal homeostasis, inhibition of cell death (apoptosis), generation of reactive radicals	Estrogens, diethylstilbestrol, synthetic androgens
9. Solid-state carcinogen	Generally only mesenchymal cells/tissues affected; physical size and shape of agent is critical; mechanism of action uncertain	Polymers (plastic), metal foils (gold), asbestos
10. Cocarcinogen	<i>Simultaneous</i> administration enhances the carcinogenic process caused by a genotoxic carcinogen; possible mechanisms include enhanced biotransformation of a procarcinogen, inhibition of detoxification of a primary carcinogen, enhanced absorption or decreased elimination of a genotoxic carcinogen	Phorbol esters, catechol, ethanol
11. Promoter	Administration <i>subsequent</i> to a genotoxic agent promotes tumor formation through enhancement of the clonal expansion of preneoplastic cells; multiple and diverse mechanisms proposed	Phorbol esters, saccharin, croton oil
12. Progressor	Development of initiated/promoted cells influenced; associated with alterations in biochemical and morphological characteristics, increased growth rate, invasiveness, and metastases; direct or indirect induction of structural (karyotypic) changes to chromosomes	Arsenic salts, benzene, hydroxyurea

Source: Adapted from Weisburger, J.H. and Williams, G.M., *Cassarett and Doull's Toxicology: The Basic Science of Poisons*, 2nd edn., Doull, J., Klaassen, C.D., and Amdur, M.O., Eds., Macmillan, New York, 1980, Chapter 6.

Additional sources: Pitot, H.C. and Dragon, Y.P., *Proc. Soc. Exp. Biol. Med.*, 202, 37, 1993; Pitot, H.C., *Chem. Ind. Inst. Toxicol.*, 13(6), 1993; Pitot, H.C., *Proc. Soc. Exp. Biol. Med.*, 198, 661, 1991; Maronpot, R.R., *Handbook of Toxicologic Pathology*, Haschek, W.M. and Rousseaux, C.G., Eds., Academic Press, San Diego, CA, 1991, Chapter 7; and Butterworth, B.E. and Goldsworthy, T.L., *Proc. Soc. Exp. Biol. Med.*, 198, 683, 1991.

^a Classifications shown are not rigid. For example, a chemical may be both genotoxic and mitogenic or cytotoxic; phorbol ester can be both a promoter and a cocarcinogen.

SPONTANEOUS CARCINOGENESIS

Spontaneous carcinogenesis occurs in rodents as well as in humans. A degree of background tumor formation is always observable in control animals from chronic rodent carcinogenicity studies. Certain organs/tissues seem to be more

susceptible to spontaneous tumor formation than others. The incidence of spontaneous tumor formation in these organs varies by species and strain. It is of interest that organs most susceptible to spontaneous tumor formation are not always the ones most susceptible to chemically induced

carcinogenesis as occurs in chronic rodent bioassays (see the section “Chemical Carcinogenesis”).

A chemical is considered to be carcinogenic in a chronic rodent study when it causes the formation of tumors in excess of background or produces a unique tumor not believed to occur spontaneously in the strain of rodent studied. Therefore, knowledge of the incidence of spontaneous tumor formation is essential for interpreting the results of chronic rodent bioassays. This section provides an overview of spontaneous tumor formation reported for several strains of mice and rats routinely used in carcinogenicity studies (Tables 14.4 through 14.9). This information is presented as a reference of spontaneous tumor formation likely to be encountered in chronic rodent studies. Because many factors

such as diet, housing conditions, or duration of study influence spontaneous tumor formation,⁵ the reader is cautioned that it would be inappropriate to use the data presented here to draw conclusions about the carcinogenic potential of any chemical. Information on spontaneous tumor formation from which such conclusions are drawn should most appropriately be obtained from concurrent controls and historical data for the specific species and strain used, as developed in and maintained by the laboratory in which the study was conducted.

The tumor incidence data provided in this section are not specific for tumor type. The reader is referred to the cited sources for more detailed descriptions of spontaneous tumor formation in the species and strains presented.

TABLE 14.4
Reported Percent Incidence^a of Spontaneous Tumor Formation by Organ/Tissue in Various Mouse Strains

Organ/Tissue	CD-1		B6C3F1	
	Male	Female	Male	Female
Adrenal	0–27.9 (%)	0–3.8	<1.0–1.4	<1.0
Body cavities	—	—	<1.0	<1.0
Brain	—	0–2.0	<0.1–0.1	0–0.1
Circulatory system	—	—	<1.0–2.9	<1.0–2.4
Heart	—	—	0.1–<1.0	0–0.1
Intestines	—	—	<1.0	<1.0
Kidney	0–2.8	0–1.4	<1.0	<0.1–<1.0
Leukemia/lymphoma	0–8.6	1.4–25.0	1.6–19.0	1.7–33.2
Liver	0–17.3	0–7.1	15.6–40.1	2.5–10.5
Lung/trachea	0–26.0	0–38.6	9.2–22.5	3.5–7.1
Mammary gland	—	0–7.3	—	<1.0–1.3
Ovary	NA	0–4.8	NA	<1.0
Pancreas	—	—	0.1–2.1	<0.1–<1.0
Pancreatic islets	0–2.1	0–1.4	<1.0	<1.0
Pituitary	0–0.8	0–10.0	<1.0	3.2–13.1
Skin/subcutaneous	0–2.8	0–2.0	<0.1–1.9	0.1–1.6
Stomach	0–4.9	0–3.8	0.3–1.1	<1.0
Testes ^b	0–2.0	NA	<1.0	NA
Thyroid	0–2.0	—	1.0–1.1	<1.0–1.7
Urinary bladder	0–2.0	0–1.4	0–0.1	<0.1–1.0
Uterus/vagina	NA	0–13.3	NA	1.2–1.9

Source: Adapted from Gad, S.C. and Weil, C.S., *Statistics and Experimental Design for Toxicologists*, Telford Press, Caldwell, NJ, 1986.

Additional sources: Chu, K., *Percent Spontaneous Primary Tumors in Untreated Species Used at NCI for Carcinogen Bioassays*, NCI Clearing House, 1977;* Fears, T.R. et al., *Cancer Res.*, 27, 1941, 1977;* Page, N.P., *Environmental Cancer*, Kraybill, H.F. and Mehlman, M.A., Eds., Hemisphere, New York, 1977, pp. 87–171;* Gart, J.J. et al., *J. Natl. Cancer Inst.*, 62, 957, 1979;* Tarone, R.E. et al., *J. Natl. Cancer Inst.*, 66, 1175, 1981;* Rao, G.N. et al., *Toxicol. Pathol.*, 18, 71, 1990; and Lang, P.L., *Spontaneous Neoplastic Lesions in the Crl:CD1[®] (ICR) BR Mouse*, Charles River Laboratories, Wilmington, MA, 1987. (*, cited in Gad and Weil [1986]).

^a Range.

^b Includes prostate and seminal vesicles.

TABLE 14.5
Reported Percent Incidence^a of Spontaneous Tumor Formation by Organ/Tissue
in Various Rat Strains

Organ/Tissue	F344		Sprague-Dawley		Wistar	
	Male	Female	Male	Female	Male	Female
Adrenal	2.4–38.1 (%)	4.0–12.0	1.4–7.6	2.7–4.3	0–48.6	0–57.1
Body cavities	<1.0–9.0	0.3–1.9	1.1–1.4	1.8	—	—
Brain	0.8–8.1	<1.0	1.4–2.7	0.9–1.6	0–8.0	0–6.0
Circulatory system	0.4–3.8	<1.0	0.5	—	0–3.3	0–2.5
Heart	<1.0	<1.0	—	—	0	0
Intestines	<1.0	<1.0	—	0.5	0–3.1	0–3.8
Kidney	<1.0	<1.0	1.6	0.9	0–2.5	0–2.0
Leukemia/lymphoma	6.5–48.0	2.1–24.6	1.9–2.2	1.4–1.6	0–12.0	0–16.0
Liver	0.5–3.4	0.5–3.9	1.1	0.5–2.2	0–5.0	0–12.0
Lung/trachea	<1.0–3.0	<1.0–2.0	1.6	2.2	0–5.7	0–2.1
Mammary gland	0–1.5	8.5–41.0	0.5–2.3	36.4–45.1	0–6.7	1.3–45.0
Ovary	NA	<1.0	NA	1.1	NA	0–4.3
Pancreas	0.2–6.0	0	—	—	0–51.7	0–1.7
Pancreatic islets	0.8–4.9	0.8–1.3	0.9–2.7	0.5	0–25.0	0–4.0
Pituitary	4.7–34.7	0.3–58.6	11.2–33.2	37.3–57.6	2.3–58.3	6.7–68.0
Preputial gland	1.4–2.4	1.2–1.8	—	—	—	—
Skin/subcutaneous	5.7–7.8	2.5–3.2	2.8–6.5	3.2–3.8	0–21.9	0–5.0
Stomach	<1.0	<1.0	—	—	0	0–2.2
Testes ^b	2.3–90.0	NA	4.2–4.3	NA	0–22.0	NA
Thyroid	3.6–12.0	4.7–10.0	1.9–3.8	1.8	0–21.7	2.5–22.4
Urinary bladder	<1.0	<1.0	0.5	—	0–2.0	0–2.0
Uterus/vagina	NA	5.5–24.6	NA	3.3–4.5	NA	1.1–25.3

Source: Adapted from Gad, S.C. and Weil, C.S., *Statistics and Experimental Design for Toxicologists*, Telford Press, Caldwell, NJ, 1986.

Additional sources: Chu, K., Percent Spontaneous Primary Tumors in Untreated Species Used at NCI for Carcinogen Bioassays, NCI Clearing House, 1977;* Fears, T.R. et al., *Cancer Res.*, 27, 1941, 1977;* N.P., *Environmental Cancer*, Kraybill, H.F. and Mehlmán, M.A., Eds., Hemisphere, New York, 1977, 87–171;* Gart, J.J. et al., *J. Natl. Cancer Inst.*, 62, 957, 1979;* Tarone, R.E. et al., *J. Natl. Cancer Inst.*, 66, 1175, 1981;* Goodman, D.G. et al., *Toxicol. Appl. Pharmacol.*, 48, 237, 1979;* Bomhard, E. et al., *J. Environ. Pathol. Toxicol. Oncol.*, 7, 35, 1986; Walsh, K.M. and Poteracki, J., *Fundam. Appl. Toxicol.*, 22, 65, 1994; Haseman, J.K., *Fundam. Appl. Toxicol.*, 3, 1, 1983; Rao, G.N. et al., *Toxicol. Pathol.*, 18, 61, 1990; and Poteracki, J. and Walsh, K.M., *Toxicol. Sci.*, 45, 1, 1998. (*, cited in Gad and Weil [1986]).

^a Range.

^b Includes prostate and seminal vesicles.

TABLE 14.6

Tumor Classification and Background Rates in F344 Rats and B6C3F1 Mice

Site	Background Rate (%)				Site	Background Rate (%)			
	Mice		Rats			Mice		Rats	
	F	M	F	M		F	M	F	M
1. Skin, breast papilloma	0.1	0.3	0.5	1.8	52. Lung squamous carcinoma	0.0	0.0	0.1	0.2
2. Respiratory, oral papilloma	0.0	0.0	0.2	0.2	53. Oral, GI squamous carcinoma	0.1	0.2	0.1	0.2
3. GI papilloma	0.8	0.6	0.2	0.2	54. Urinary, reproductive squamous carcinoma	0.0	0.0	0.2	0.1
4. Urinary, reproductive papilloma	0.1	0.1	0.2	0.2	55. Skin, GI basal cell carcinoma	0.1	0.0	0.2	0.4
5. Skin, breast adenoma	1.5	1.8	0.9	0.1	56. Urinary transitional cell carcinoma	0.0	0.0	0.1	0.1
6. Respiratory, oral adenoma	3.8	9.8	1.2	1.8	57. Skin, breast adenocarcinoma	1.3	0.1	1.9	0.2
7. Liver adenoma	3.6	9.7	0.1	0.1	58. Lung adenocarcinoma	0.0	0.0	0.0	0.0
8. GI adenoma	0.4	0.7	1.0	4.5	59. Oral, GI adenocarcinoma	0.1	0.5	0.1	0.5
9. Urinary, reproductive adenoma	0.3	0.1	1.9	2.4	60. Urinary, reproductive adenocarcinoma	0.3	0.0	1.1	0.3
10. Pituitary adenoma	8.3	0.4	26.5	15.8	61. Endocrine, brain adenocarcinoma	0.1	0.1	0.5	0.2
11. Endocrine adenoma	0.5	1.2	0.3	0.7	62. Islet cell carcinoma	0.1	0.0	0.2	1.2
12. Skin, urinary adenoma	0.0	0.1	0.0	0.2	63. Bile duct carcinoma	0.0	0.0	0.0	0.0
13. Reproductive, endocrine adenoma	0.1	0.0	0.1	0.2	64. Hepatocellular carcinoma	3.6	18.9	2.1	3.1
14. Tubular cell adenoma	0.0	0.1	0.1	0.2	65. Alveolar, broncheolar carcinoma	1.8	5.6	0.4	0.9
15. Follicular, clear cell adenoma	1.9	1.2	6.2	6.9	66. Chromophobe carcinoma	0.1	0.0	0.4	0.2
16. Cortical adenoma	0.4	1.2	2.3	1.0	67. Tubular cell adenocarcinoma	0.0	0.1	0.0	0.2
17. Skin, breast, liver cystadenoma	0.1	0.3	0.4	0.0	68. Thyroid follicular cell carcinoma	0.3	0.2	0.5	0.9
18. GI, urinary, reproductive cystadenoma	0.3	0.0	0.0	0.0	69. Cortical carcinoma	0.0	0.1	0.2	0.1
19. Endocrine cystadenoma	0.1	0.0	0.1	0.0	70. Clear cell carcinoma	0.0	0.0	2.4	2.8
20. Acinar cell adenoma	0.0	0.0	0.1	1.4	71. Adnexal, sebaceous carcinoma	0.0	0.0	0.2	0.3
21. Keratoacanthoma	0.1	0.1	0.3	1.6	72. Thymoma	0.0	0.0	0.0	0.0
22. Tubular adenoma	0.4	0.0	0.2	0.0	73. Granulosa cell carcinoma	0.3	NA	0.5	NA
23. Interstitial cell tumor	NA	0.4	NA	83.3	74. Interstitial cell carcinoma	NA	0.0	NA	1.0
24. Pheochromocytoma	0.9	1.4	4.4	18.6	75. Pheochromocytoma, malignant	0.1	0.1	0.4	1.6
25. Skin, breast fibroma	0.0	1.2	1.4	4.5	76. Skin sarcoma	1.2	4.6	0.8	1.7
26. Blood, bone fibroma	0.0	0.0	0.0	0.1	77. Other sites sarcoma	0.3	0.2	0.4	0.5
27. Fibroma, other sites	0.0	0.0	0.3	0.0	78. Blood, bone sarcoma	0.3	0.3	0.2	0.6
28. Lipoma	0.2	0.1	0.3	0.7	79. Liposarcoma	0.0	0.0	0.0	0.1
29. Leiomyoma	0.3	0.0	0.2	0.1	80. Leiomyosarcoma	0.6	0.3	0.4	0.2
30. Endometrial stromal polyp	1.3	0.0	17.9	0.0	81. Endometrial stromal sarcoma	0.3	0.0	1.0	0.1
31. Fibroadenoma	0.2	0.0	22.4	2.0	82. Carcinosarcoma	0.0	0.1	0.0	0.1
32. Hemangioma	1.1	1.2	0.1	0.2	83. Mesothelioma, osteosarcoma	0.4	0.3	0.4	3.2
33. Osteoma	0.1	0.0	0.0	0.1	84. Teratoma	0.1	0.1	0.0	0.0
34. Hamartoma	0.0	0.0	0.0	0.0	85. Hemangiosarcoma	1.7	2.6	0.1	0.3
35. Ganglioneuroma	0.0	0.1	0.3	0.5	86. Granular cell tumor	0.0	0.0	0.1	0.1
36. Chromophobe adenoma	1.3	0.0	12.2	5.0	87. Glioma	0.0	0.0	0.2	0.3
37. Skin, breast carcinoma	0.1	0.1	0.3	0.4	88. Oligodendroglioma	0.0	0.0	0.2	0.2
38. Blood, bone carcinoma	0.1	0.0	0.1	0.1	89. Astrocytoma	0.0	0.0	0.5	0.5
39. Lung carcinoma	0.0	0.0	0.0	0.0	90. Olfactory neuroblastoma	0.0	0.0	0.0	0.0
40. Oral, GI carcinoma	0.0	0.1	0.0	0.0	91. Neurofibrosarcoma	0.2	0.4	0.3	0.5
41. Urinary carcinoma	0.0	0.0	0.0	0.0	92. Lymphoma	8.3	3.4	1.4	1.7
42. Reproductive carcinoma	0.1	0.0	1.5	1.7	93. Lymphocytic lymphoma	3.8	1.6	0.3	0.3
43. Pituitary carcinoma	0.4	0.0	2.2	1.1	94. Histiocytic lymphoma	4.8	2.6	0.2	0.3
44. Endocrine carcinoma	0.0	0.0	0.0	0.1	95. Mixed lymphoma	6.2	2.3	0.0	0.0
45. Brain carcinoma	0.1	0.0	0.4	0.1	96. Malignant reticulosis	0.1	0.1	0.0	0.0
46. Skin, breast papillary carcinoma	0.1	0.0	0.1	0.0	97. Leukemia	0.4	0.2	3.1	4.3
47. Lung papillary carcinoma	0.0	0.0	0.0	0.0	98. Myelomonocytic leukemia	0.0	0.0	1.7	2.1
48. GI, urinary papillary carcinoma	0.0	0.0	0.0	0.0	99. Lymphocytic leukemia	0.5	0.1	0.8	1.3
49. Uterus, ovary papillary carcinoma	0.0	NA	0.1	NA	100. Plasmacytic leukemia	0.1	0.1	7.5	10.9
50. Thyroid papillary carcinoma	0.0	0.0	0.0	0.1	101. Granulocytic leukemia	0.1	0.1	0.2	0.2
51. Skin squamous carcinoma	0.1	0.2	0.7	1.2	102. Monocytic leukemia	0.0	0.0	1.8	2.2

Source: Linkov, I. et al., *Toxicol. Sci.*, 43, 1, 1998, with permission from Oxford University Press.

Notes: The information in this table was developed from the NCI/NTP Carcinogenesis Bioassay Data Base (CBDS) system and includes only experiments completed by 1983. Rates are averages in all control groups in relevant experiments (for 312 chemicals) and are rounded to one decimal place. Some designations include multiple sites for the subject tumor.

TABLE 14.7
Spontaneous Neoplasms Occurring in Three or More Control Wistar Rats

Neoplasm	Male			Female		
	No.	% ^a	% Range	No.	% ^a	% Range
<i>Endocrine</i>						
Pituitary adenoma	156	33.6	18.3–51.7	234	50.3	43.1–58.3
Carcinoma				10	2.2	0–5.4
Adrenal pheochromocytoma—benign	45	9.7	4.0–21.7	6	1.3	0–3.3
Pheochromocytoma—malignant	8	1.7	0–3.3	2	0.4	0–1.0
Cortical adenoma	16	3.4	0–9.5	19	4.1	0–7.0
Cortical carcinoma	1	0.2	0–1.0	4	0.9	0–1.7
Thyroid C-cell adenoma	27	5.8	3.3–11.4	39	8.4	5.8–10.0
C-cell carcinoma	2	0.4	0–0.8	2	0.4	0–2.0
Follicular adenoma	18	3.9	1.7–6.9	13	2.9	2.0–3.3
Follicular carcinoma	4	0.9	0–1.7	7	1.5	0–3.3
Pancreatic islet cell adenoma	25	5.4	0–25.0	8	1.7	0.8–4.0
Islet cell carcinoma	7	1.5	0–6.7	3	0.7	0–1.5
Parathyroid adenoma	9	1.9	0–4.0			
<i>Integumentary</i>						
Mammary fibroadenoma	12	2.6	0–6.7	168	36.1	18.0–45.0
Adenoma				18	3.9	2.0–6.7
Adenocarcinoma				31	6.7	1.7–12.4
Keratoacanthoma	52	11.2	2.0–21.9	3	0.7	0–1.9
Fibroma	21	4.5	1.0–9.2	14	3.0	1.9–5.0
Fibrosarcoma	15	3.2	0.8–6.2			
Papilloma	15	3.2	1.0–8.3	2	0.4	0–0.8
Squamous cell carcinoma	6	1.3	0–3.3	5	1.1	0–3.3
Lipoma	5	1.1	0–4.0			
Liposarcoma	2	0.4	0–1.5	1	0.2	0–0.8
Trichofolliculoma	7	1.5	0–4.2	1	0.2	0–0.8
Pilomatrixoma	2	0.4	0–1.0	1	0.2	0–1.0
Schwannoma	2	0.4	0–0.8	2	0.4	0–2.0
Basal cell tumor	3	0.7	0–2.0	3	0.7	0–2.0
Histiocytic sarcoma	3	0.7	0–1.9			
Zymbal's gland carcinoma	3	0.7	0–1.7	1	0.2	0–0.8
<i>Reproductive</i>						
Testicular interstitial cell tumor	49	10.5	8.3–15.2			
Prostatic adenoma	3	0.7	0–1.5			
Uterine/vaginal stromal polyp				72	15.5	7.7–20.8
Stromal sarcoma				5	1.1	0–4.0
Schwannoma				11	2.4	0–5.0
Granular cell tumor				13	2.8	0–6.2
Endometrial adenoma				4	0.9	0–3.1
Granulosa cell tumor				7	1.5	1.0–2.0
<i>Gastrointestinal</i>						
Pancreatic acinar cell adenoma	62	13.3	0–51.7	3	0.7	0–1.7
Acinar cell carcinoma	16	3.4	0–16.7			
Hepatocellular adenoma	13	2.8	0–5.0	10	2.2	0–4.2
Carcinoma	5	1.1	0–2.0	1	0.2	0–0.08
Cholangioma				3	0.7	0–2.0
Intestinal adenocarcinoma	6	1.3	0–3.1	4	0.9	0–2.3
Intestinal leiomyoma	2	0.4	0–1.7	4	0.9	0–2.3
<i>Hematopoietic/lymphatic</i>						
Thymoma—benign	14	3.0	0–5.0	35	7.5	0–14.6
Thymoma—malignant	2	0.4	0–2.0	5	1.1	0–3.3
Lymphoma	6	1.3	0–4.6	11	2.4	0–7.7

TABLE 14.7 (continued)
Spontaneous Neoplasms Occurring in Three or More Control Wistar Rats

Neoplasm	Male			Female		
	No.	% ^a	% Range	No.	% ^a	% Range
Granular cell leukemia	5	1.1	0–2.0	7	1.5	0–3.1
Histiocytic sarcoma	5	1.1	0–3.1	3	0.7	0–1.7
<i>Nervous</i>						
Granular cell meningioma	11	2.4	0–3.3	7	1.5	0–3.1
Astrocytoma	4	0.9	0–2.0	3	0.7	0–1.5
Oligodendroglioma	2	0.4	0–1.0	2	0.4	0–1.0
<i>Urinary</i>						
Renal cell adenoma	8	1.7	0–2.5	3	0.7	0–1.5
Renal cell carcinoma	2	0.4	0–0.8	1	0.2	0–0.8
Renal mesenchymal tumor	4	0.9	0–1.7	11	2.4	0–3.8
<i>Cardiovascular</i>						
Cardiac Schwannoma	2	0.4	0–1.5	5	1.1	0–2.0
Hemangioma	8	1.7	0–3.3	6	1.3	0–1.5
Hemangiosarcoma	16	3.4	0–3.3	3	0.7	0–2.5
<i>Musculoskeletal</i>						
Osteosarcoma	1	0.2	0–2.0	3	0.7	0–2.0

Source: Poteracki, J. and Walsh, K.M., *Toxicol. Sci.*, 45, 1, 1998 with permission from Oxford University Press.

Note: This table reports neoplasms from 930 control rats (465 male, 465 female) from five carcinogenicity bioassays conducted between 1990 and 1995.

^a Percent of population (male or female), range is between studies.

TABLE 14.8
Ranking of Mouse Organs Based on Incidence of Spontaneous Tumor Formation (Benign and Malignant)

Males		Females	
Organ	Incidence ^a	Organ	Incidence ^a
1. Liver	28.7 (%)	1. Blood/lymphoid tissue ^b	29.1
2. Lung/trachea	24.3	2. Lung/trachea	22.9
3. Adrenal	14.7	3. Pituitary	11.6
4. Blood/lymphoid tissue ^b	13.8	4. Liver	8.8
5. Stomach	3.0	5. Uterus/vagina	7.6
6. Circulatory system	2.9	6. Mammary gland	4.3
7. Skin/subcutaneous	2.4	7. Ovary	2.8
8. Pancreas	2.1	8. Circulatory system	2.4
9. Thyroid	1.6	9. Adrenal	2.2
10. Kidney	1.5	10. Stomach	2.1
11. Pancreas (islets)	1.2	11. Skin/subcutaneous	1.8
Testes	1.2	12. Thyroid	1.7
12. Urinary bladder	1.1	13. Kidney	1.4
13. Pituitary	0.5	14. Urinary bladder	1.2
14. Intestines	0.4	15. Brain	1.1
15. Brain	0.1	16. Pancreas	<1.0
Heart	0.1	Pancreas (islets)	0.8
Body cavities	0.1	17. Body cavities	0.3
		18. Intestines	0.2
		19. Heart	0.1

^a Mean of highest reported percent incidence of spontaneous tumor formation for various mouse strains.

^b Leukemia/lymphoma.

TABLE 14.9
Ranking of Rat Organs Based on Incidence of Spontaneous Tumor Formation (Benign and Malignant)

Males		Females	
Organ	Incidence ^a	Organ	Incidence ^a
1. Pituitary	42.1(%)	1. Pituitary	61.4
2. Testes	38.8	2. Mammary gland	43.7
3. Adrenal	31.4	3. Adrenal	24.5
4. Pancreas	28.9	4. Uterus/vagina	18.1
5. Blood/lymphoid tissue ^b	20.7	5. Blood/lymphoid tissue ^b	14.1
6. Thyroid	12.5	6. Thyroid	11.4
7. Skin/subcutaneous	12.1	7. Liver	6.0
8. Pancreas (islets)	10.9	8. Skin/subcutaneous	4.0
9. Brain	6.3	9. Brain	2.7
10. Body cavities	3.5	10. Lung/trachea	2.1
Mammary gland	3.5	11. Pancreas (islets)	1.9
11. Lung/trachea	3.4	Ovary	1.9
12. Liver	3.2	Intestines	1.9
13. Circulatory system	2.5	12. Preputial gland	1.8
14. Preputial gland	2.4	Circulatory system	1.8
15. Intestines	2.1	13. Pancreas	1.7
16. Kidney	1.7	14. Body cavities	1.4
17. Stomach	1.2	15. Urinary bladder	1.1
18. Urinary bladder	0.9	Stomach	1.1
19. Heart	0.2	16. Kidney	1.0
		17. Heart	<0.1

^a Mean of highest reported percent incidence of spontaneous tumor formation for various rat strains.

^b Leukemia/lymphoma.

CHEMICAL CARCINOGENESIS

The chronic rodent carcinogenicity study is one of the most elaborate, labor-intensive, and costly of all toxicology studies. To evaluate the carcinogenic potential of chemicals, animals are exposed to a substance of concern throughout their lifetime during which time the appearance of unique and/or excess tumors are noted. Although some tumors are observable grossly, the ultimate identification and morphological characterization are made through histopathologic examination of generally 45 organs/tissues per animal. Considering the typical study consists of 400 rodents, as many as 9000 microscopic sections can be evaluated just in the control and high-dose groups. In addition, data collected on physical examinations, body weights, food consumption, and necropsy during as many as 24 months must be compiled and analyzed. The cost of a chronic carcinogenicity study in one species can be US \$1.5 million or higher depending on the route

of exposure. The seriousness of the endpoint evaluated is usually justification for the time and money expended since in the eyes of the public, cancer is the most feared of all human diseases.

Widespread and routine evaluation of chemicals for carcinogenic potential has been performed for over five decades beginning in earnest in the mid-1960s.¹ Despite many problems associated with the chronic rodent carcinogenicity study, discussion of which is beyond the scope of this book, the rodent bioassay is still considered the best available method for identifying potential carcinogens.¹ Most of the substances known to be carcinogenic to humans have been found to cause cancer in animals.⁶ The converse of this, that most of the substances shown to cause cancer in animals will cause cancer in humans under realistic conditions of exposure, is the subject of much debate.

As pointed out by Maronpot,¹ the original National Cancer Institute (NCI) chronic rodent carcinogenicity bioassay was intended to screen for potential carcinogens and

was not intended to define carcinogen potency, mechanisms of tumor formation, or human relevance. However, results of these studies are often used in this way for human risk assessment. The reader should bear in mind that these studies only demonstrate that certain chemicals are capable of causing cancer in animals under specific conditions of exposure. The judgment as to whether a chemical poses a carcinogenic risk should only be made after factors such as route and duration of exposure, genotoxic potential, comparative species metabolism, as well as other information relevant to human exposure are considered.¹

This section presents information on the basic design of the chronic rodent carcinogenicity study and the reported susceptibility of various organs/tissues of rodents to chemically induced tumor formation (Tables 14.10 through 14.17). The reader is referred to the cited sources for more detailed discussion of the chronic rodent carcinogenicity study, its conduct, and interpretation.

The 2 year rodent bioassay has been the “gold standard” for evaluation of chemical carcinogenicity for over 30 years. However, it has come under criticism because of its high levels of spontaneous tumors, high levels of mortality (even in control animals), large number of animals, low sensitivity, and questionable relevance to humans. Beginning in the 1990s, alternative carcinogen bioassays which use transgenic (Tg) mice were designed to address these concerns and proposed as replacements to the 2 year mouse bioassay for evaluation of pharmaceuticals. Validation studies were organized by the US National Toxicology Program (NTP) and ILSI/HESI studies. Early papers discussed the sensitivity and utility of the *p53*^{+/-} and Tg.AC models⁴² and the Tg.*rasH2*⁴³ model. Details of the construction and validation of these models are beyond the scope of this chapter but can be found in a number of review articles.⁷⁻⁹

The ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) Guideline S1B issued in 1997 permitted the use of these “alternative short-term” carcinogenicity assays.¹⁰ Initially in the United States, the *p53*^{+/-} and TG.AC models were used mainly based on availability and familiarity of the models to the US FDA.¹¹ The *p53*^{+/-} mouse has one allele of the *p53* tumor suppressor gene inactivated. The *p53*^{+/-} model only responds to genotoxic agents. The TG.AC and Tg.*rasH2* mice have a transgene carrying a mutated human *ras* oncogene inserted into every cell of the animals. The TG.AC is a dermal model used initially for nongenotoxic agents. The Tg.*rasH2* responds to both genotoxic and nongenotoxic carcinogens.¹¹ The Tg.*rasH2* has gradually become the preferred model following its availability in the United States. The *p53*^{+/-} mouse is available but its use must be justified by demonstrating that the test article is in fact genotoxic. After the Tg.*rasH2* mouse began to be used for all nondermal drugs, the TG.AC mouse was used for

a period of time for dermal drugs but is no longer recommended by FDA and is no longer being bred. As this book goes to press, about 25% of the mouse carcinogenicity protocols submitted to the FDA-CDER's Executive Carcinogen Assessment Committee are transgenic studies.⁸ In Europe, there have been recommendations to use the alternative transgenic mouse carcinogenicity models for REACH testing.^{8,12}

Comparisons of the available data in both standard 2 year and transgenic bioassay models show that substitution of a transgenic mouse short-term carcinogenicity bioassay has resulted in a net reduction of positive findings for non-carcinogens.^{9,11,13} The transgenic mouse models allow the use of fewer animals due to high survival to the end of the 26 week study and lower spontaneous tumor background.⁹ The shorter duration of the study is the result of accelerated tumorigenesis in these transgenic models relative to the wild type.⁹ Inclusion of a positive control group, treated with a known carcinogen active in the particular model, demonstrates the responsiveness of the transgene.¹³ As the use of the transgenic mouse models grow, particularly the Tg.*rasH2* mouse used in regulatory submissions, historical control data enumerating spontaneous tumor incidence will lend additional support to their use to replace the 2 year mouse bioassay where possible.

Study designs for alternative short-term transgenic mouse carcinogenicity studies are smaller in size and shorter than traditional 2 year studies. Dose range-finding studies usually include a 5 day preliminary toxicity study followed by a 28 day toxicity/TK study. This is in comparison to a 14 day preliminary toxicity study and a 90 day toxicity/TK study usually used for dose selection for a 2 year carcinogenicity study. The main transgenic carcinogenicity study involves daily dosing for 6 months as opposed to 2 years for a standard study. Group sizes for the transgenic carcinogenicity study are 25 per sex per dose as opposed to group sizes of 50, 60, or even 75 for a standard 2 year mouse carcinogenicity study. The smaller, shorter phases associated with the Tg.*rasH2* assay result in the use of fewer animals, use of less test article, lower facility requirements, and less staff requirements. This permits quicker and cheaper testing. Should a positive result be obtained, this can be a year or more before the 2 year rat study is completed.

Oral gavage is the most common route of administration followed by dosed feed. Lower numbers of intradermal subcutaneous, intermittent IV dosing, and inhalation studies have also been run. The Tg.*rasH2* mouse is available from Taconic (Germantown, NY) in the United States and CLEA (Kawasaki City, Japan). The toxicity and TK studies are usually performed with nontransgenic B6C3F1 littermates that result from the breeding of Tg.*rasH2* mice. The actual carcinogenicity studies are run using the transgenic Tg.*rasH2* mice (Table 14.18).

TABLE 14.10
Basic Design of a Chronic Carcinogenicity Study^a

Animals	
Species	Usually rats, mice, and occasionally hamsters
Strain	Common laboratory strains (e.g., rats: F344, Sprague-Dawley mice: CD-1, B ₆ C ₃ F ₁)
Age	<6–8 weeks
Housing	Individual or group
Study Design	
No. animals/group	50/sex/group ^b
No. groups	3 dose levels/1 control ^c
Exposure route	Oral, dermal, or inhalation as appropriate to potential human exposure
Study duration	Average lifespan of strain used, at least 18 months for mice, 24 months for rats
Observations	
Mortality/morbidity	2 times/day
Gross clinical observations	At least daily
Detailed physical examinations	1 time/week for first 3 months then once every 2–4 weeks as study progresses. Palpation monthly to weekly during the latter two-third of the study
Body weights	Weekly (weeks 1–13), monthly thereafter
Food consumption	Weekly (weeks 1–13), monthly thereafter
Water consumption	Weekly (weeks 1–13), monthly thereafter (when test substance is administered in drinking water)
Clinical pathology	Currently recommended is collection of blood smears at scheduled and unscheduled sacrifices of main study animals to be examined only if indicated to aid in the diagnosis of possible hematopoietic neoplasia following histopathologic evaluation ^d
Ophthalmoscopic examination	Pretest and at termination (not always performed)
Gross necropsy	All animals (including spontaneous deaths)
Organ weights	Not always performed
Histopathology	All animals. See Table 14.11 for organs/tissues frequently observed

^a Based in part on US Environmental Protection Agency Guidelines.³³

^b Additional animals per group if interim sacrifices are planned and for toxicokinetic determinations.

^c Additional control groups may be needed depending on known or suspected toxicity of the vehicle used. Dose levels should be spaced to produce a gradation of effects. The highest dose should elicit signs of toxicity without substantially altering the normal lifespan due to effects other than tumors. The lowest dose level should produce no evidence of toxicity. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects.

^d Young et al.³⁴

TABLE 14.11
Organs/Tissues Typically Selected for Histopathologic Examination in Chronic Carcinogenicity Studies

Adrenals	Kidney(s)	Spleen
Aorta	Lacrimal gland ^a	Sternum
Bone marrow (sternal/femoral)	Liver	Stomach
Brain	Lung(s)	Testes
Medulla/pons	Lymph nodes (representative)	Thymus
Cerebellum		Thyroid
Cerebral cortex	Mammary gland	Trachea
Esophagus	Nasal turbinates ^a	Urinary bladder
Eye(s)	Nerve (peripheral)	Uterus
Femur	Ovaries	Vagina ^a
Gall bladder (if applicable)	Pancreas	Zymbal's gland ^a
Harderian gland ^a	Parathyroids	Other
Heart	Pituitary	Accessory genital organs
Intestines	Salivary gland	All gross lesions/tumors
Duodenum	Skeletal muscle	
Jejunum	Skin	
Ileum	Spinal cord	
Cecum	Cervical	
Colon	Midthoracic	
Rectum	Lumbar	

^a Occasionally selected.

TABLE 14.12
National Toxicology Program's Levels of Evidence of Carcinogenicity Used for Interpretative Conclusions Regarding Chronic Rodent Carcinogenicity Study Results
Clear evidence of carcinogenic activity

Demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy

Some evidence of carcinogenic activity

Demonstrated by studies that are interpreted as showing a chemically related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence

Equivocal evidence of carcinogenic activity

Demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related

No evidence of carcinogenic activity

Demonstrated by studies that are interpreted as showing no chemically related increases in malignant or benign neoplasms

Inadequate study of carcinogenic activity

Demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity

Source: Maronpot, R.R., *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, 1991. With permission.

TABLE 14.13
Top Ten Organs/Systems Developing Tumors in Carcinogenesis Studies—Mice^a

Ranking	Males	Females
1	Liver	Liver
2	Lung	Lung
3	Forestomach	Forestomach
4	Circulatory system	Hematopoietic system
5	Hematopoietic system	Circulatory system
6	Thyroid gland	Mammary gland
7	Harderian gland	Ovary
8	Adrenal gland	Thyroid gland
9	Kidney	Uterus/cervix
10	Five sites ^b	Harderian gland

Source: Modified from Huff, J. et al., *Environ. Health Perspect.*, 93, 247, 1991.

Note: See Table 22.14 for a ranking of human organs/systems based on frequency of occurrence of site-specific cancers.

^a Based on 379 long-term chemical carcinogenesis studies from the National Toxicology Program (NTP) data base.

^b Heart, nasal cavity, preputial gland, skin, and urinary bladder.

TABLE 14.14
Top 10 Organs/Systems Developing Tumors in Carcinogenesis Studies—Rats^a

Ranking	Males	Females
1	Liver	Liver
2	Kidney	Mammary gland
3	Zymbal's gland	Zymbal's gland
4	Forestomach	Thyroid gland
5	Thyroid gland	Forestomach
6	Skin	Urinary bladder
7	Hematopoietic system	Clitoral gland
8	Urinary bladder	Hematopoietic system
9	Intestines	Kidney
10	Nasal cavity	Uterus/cervix

Source: Modified from Huff, J. et al., *Environ. Health Perspect.*, 93, 247, 1991.

Note: See Table 22.14 for a ranking of human organs/systems based on frequency of occurrence of site-specific cancers.

^a Based on 379 long-term chemical carcinogenesis studies from the National Toxicology Program (NTP) data base.

TABLE 14.15
Frequency of Carcinogenic Response to Chemicals
by Organ/System—Rats and Mice^a

	Number Positive at Site (%) ^b	
	Chemicals Evaluated as Carcinogenic in Rats (<i>n</i> = 354) ^c	Chemicals Evaluated as Carcinogenic in Mice (<i>n</i> = 299) ^c
Liver	143 (40%)	171 (57%)
Lung	31 (9%)	83 (28%)
Mammary gland	73 (21%)	14 (5%)
Stomach	60 (17%)	42 (14%)
Vascular system	26 (7%)	47 (16%)
Kidney/ureter	45 (13%)	12 (4%)
Hematopoietic system	35 (10%)	39 (13%)
Urinary bladder/urethra	37 (10%)	12 (4%)
Nasal cavity/turbinates	33 (9%)	4 (1%)
Ear/Zymbal's gland	30 (9%)	2
Esophagus	29 (8%)	7 (2%)
Small intestine	21 (6%)	3 (1%)
Thyroid gland	20 (6%)	10 (3%)
Skin	20 (6%)	1
Peritoneal cavity	17 (5%)	7 (2%)
Oral cavity	16 (5%)	1
Large intestine	15 (4%)	
Central nervous system	15 (4%)	2
Uterus	11 (3%)	5 (2%)
Subcutaneous tissue	10 (3%)	1
Pancreas	9 (3%)	
Adrenal gland	7 (2%)	4 (1%)
Pituitary gland	7 (2%)	4 (1%)
Clitoral gland	7 (2%)	2
Preputial gland	2	7 (2%)
Testes	6 (2%)	1
Harderian gland		6 (2%)
Spleen	6 (2%)	
Ovary		4 (1%)
Gall bladder		3 (1%)
Bone	3	
Mesovarium	2	
Myocardium		2
Prostate	2	
Vagina	1	

Source: Gold, L.S. et al., *Environ. Health Perspect.*, 93, 233, 1991.
 With permission.

^a Based on 354 and 299 chemicals considered carcinogenic to rats and mice, respectively, in long-term chemical carcinogenesis studies from the carcinogenic potency database (CPDB).

^b Percentages not given when fewer than 1% of the carcinogens were active at a given site.

^c Chemicals have been excluded for which the only positive results in the CPDB are for "all tumor bearing animals," that is, there is no reported target site.

TABLE 14.16
Animal Neoplastic Lesions of Questionable
Significance to Humans

- Male rat renal tumors with β_2 -globulin nephropathy
- Rodent urinary bladder neoplasia associated with calcium phosphate precipitates
- Rodent hepatocellular neoplasia with peroxisome proliferation
- Adenohypophysis neoplasia in rats with dopamine inhibitors
- Rodent thyroid follicular cell tumors resulting from adaptive hormonal mechanisms
- Splenic sarcomas in rats
- Pancreatic islet cell neoplasia in rats with neuroleptics
- Rodent stomach carcinoid tumors associated with prolonged acid secretion suppression
- Forestomach neoplasia in rats and mice
- Osteomas in mice
- Mononuclear cell leukemia in F344 rats
- Canine mammary neoplasia related to progestagen administration
- Rodent mammary neoplasia related to adaptive hormonal responses
- Rat uterine endometrial carcinomas related to dopamine agonists
- Uterine leiomyoma in mice with β_1 -antagonists
- β_2 -Receptor stimulant-induced rat mesovarian leiomyomas
- Mouse ovarian tubulostromal adenomas
- β_2 -Receptor stimulant-induced rat mesovarian leiomyomas
- Leydig cell tumors in rat and mice testes

Sources: Alison, R.H. et al., *Toxicol. Pathol.*, 22, 179, 1994; Williams, G.M. and Iatropoulos, M.J., *Principles and Methods of Toxicology*, 4th edn., Hayes, A.W., Ed., Taylor & Francis, Philadelphia, PA, 2001, Chapter 20.

TABLE 14.17**Criteria for Determining the Human Relevance of Animal Bioassay Results****Supportive**

Same exposure route as humans
 Tumors with several types of exposure
 Tumors in several species
 Tumor site correspondence
 Tumors at multiple sites
 Tumors at sites of low spontaneous occurrence
 Tumors in tissues analogous to human tissues
 No evidence of cellular toxicity at the target site
 Tumors appear early in life
 Tumors progress rapidly (benign to malignant)
 Tumors usually fatal
 Similar metabolism (biotransformation) in animals and humans
 Genotoxic
 DNA-reactive
 Mechanism of tumorigenesis relevant to humans
 Structural similarity to known human carcinogens
 No evidence for disruption of homeostasis

Not Supportive

Different exposure route than humans
 Tumors with only one type of exposure (not relevant to humans)
 Tumors in only one species
 No site correspondence across species
 Tumors at only one site
 Tumors at sites with high background incidence
 Tumors in animal tissues not relevant to humans
 Tumors only in organs displaying cellular toxicity
 Tumors detectable only late in life
 Benign tumors only
 Tumors not fatal
 Metabolic pathways differ in humans and animals
 Nongenotoxic
 No reaction with DNA
 Mechanism of tumorigenesis does not occur in humans
 Little structural similarity to known human carcinogens
 Homeostasis disrupted

Source: Modified from Ashby, J. et al., *Regul. Toxicol. Pharmacol.*, 12, 270, 1990. With permission.

TABLE 14.18**Basic Design of a Short-Term TgMouse Carcinogenicity Study^a**

Animals	
Species	Mice
Strain	CByB6F1-Tg(HRAS)2Jic (+/- hemizygous c-Ha-ras, referred to as Tg.rasH2). Wild-type litter mates CByB6F1-Tg(HRAS)2Jic (-/- homozygous c-Ha-ras, referred to as CByB6F1) are used for range-finding and toxicokinetic studies. Available only from Taconic in the United States and CIEA in Japan
Age	At least 6 weeks but no more than 10 weeks
Housing	Individual
Study Design	
No. animals/group	25/sex/group ^b
No. groups	3 dose levels/1 control, 1 positive control ^{c,d}
Exposure route	Oral, dermal, or inhalation as appropriate to potential human exposure
Study duration	26 weeks
Observations	
Mortality/morbidity	2 times a day
Gross clinical observations	At least daily
Detailed physical examinations	1 time/week includes palpation
Body weights	Weekly (weeks 1–13), every other week thereafter
Food consumption	Weekly (weeks 1–13), every other week thereafter
Water consumption	Weekly (weeks 1–13), every other week thereafter (when test substance is administered in drinking water)
Clinical pathology	None currently recommended as hematopoietic neoplasia is not generally noted

(continued)

TABLE 14.18 (continued)**Basic Design of a Short-Term TgMouse Carcinogenicity Study^a**

	Observations
Ophthalmoscopic examination	Pretest and at termination (not always performed)
Gross necropsy	All animals (including spontaneous deaths)
Organ weights	Not always performed.
Histopathology	See Table 14.11 for organs/tissues frequently observed, suspected target organs/tissues—all groups, all gross lesions and tumors

^a Based in part on ICH S1B Guideline.

^b Additional animals per group if unusual toxicity is suspected or for toxicokinetic determinations.

^c Additional control groups may be needed depending on known or suspected toxicity of the vehicle used. Dose levels should be spaced to produce a gradation of effects. The highest dose should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors. The lowest dose level should produce no evidence of toxicity. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects.

^d The positive control (a known carcinogen such as MNU or urethane) is used to evaluate the response of the transgene.

TUMOR PATHOLOGY

The primary goal of the chronic rodent carcinogenicity study is to assess the development of tumors in animals exposed to a chemical of concern as compared with controls. Four types of neoplastic responses are considered to be evidence of chemically induced carcinogenesis¹⁴: (1) a greater incidence of tumors in exposed animals than occurs spontaneously in controls; (2) the earlier

development of tumors in exposed animals than observed in controls; (3) the formation of unique tumors in exposed animals which do not occur spontaneously in controls; and (4) an increased multiplicity of tumors in exposed animals compared with controls. In the chronic rodent bioassay, the ultimate discovery and identification of any tumors must come from histopathologic examination. Basic information on tumor pathology is presented in this section (Tables 14.19 through 14.22).

TABLE 14.19**Capacity of Tissues to Undergo Hyperplasia**

High capacity
Surface epithelium
Hepatocytes
Renal tubules
Fibroblasts
Endothelium
Mesothelium
Hematopoietic stem cells
Lymphoid cells
Moderate capacity
Glandular epithelium
Bone
Cartilage
Smooth muscle of vessels
Smooth muscle of uterus
Low capacity
Neurons
Skeletal muscle
Smooth muscle of GI tract

Source: Maronpot, R.R., *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, 1991. With permission.

TABLE 14.20
Selected Examples of Presumptive Preneoplastic Lesions

Tissue	Presumptive Preneoplastic Lesion^a
Mammary gland	Hyperplastic alveolar nodules (HANs), atypical epithelial proliferation, lobular hyperplasia, intraductal hyperplasia, hyperplastic terminal duct
Liver	Foci of cellular alteration, hepatocellular hyperplasia, oval cell proliferation, cholangiofibrosis
Kidney	Karyocytomegaly, atypical tubular dilation, atypical tubular hyperplasia
Skin	Increase in dark basal keratinocytes, focal hyperplasia/hyperkeratosis
Pancreas (exocrine)	Foci of acinar cell alteration, hyperplastic nodules, atypical acinar cell nodules

Source: Maronpot, R.R., *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, 1991. With permission.

^a Many of these presumptive preneoplastic lesions are seen in carcinogenicity studies utilizing specific animal model systems. Generalizations about these presumptive preneoplastic lesions are inappropriate outside the context of the specific animal model system being used.

TABLE 14.21
Comparative Features of Benign and Malignant Neoplasms

	Benign	Malignant
General effect on the host	Little; usually do not cause death	Will almost always kill the host if untreated
Rate of growth	Slow; may stop or regress	More rapid (but slower than "repair" tissue); autonomous; never stop or regress
Histological features	Encapsulated; remain localized at primary site	Infiltrate or invade; metastasize
Mode of growth	Usually grow by expansion, displacing surrounding normal tissue	Invade, destroy, and replace surrounding normal tissue
Metastasis	Do not metastasize	Most can metastasize
Architecture	Encapsulated; have complex stroma and adequate blood supply	Not encapsulated; usually have poorly developed stroma; may become necrotic at center
Danger to host	Most without lethal significance	Always ultimately lethal unless removed or destroyed <i>in situ</i>
Injury to host	Usually negligible but may become very large and compress or obstruct vital tissue	Can kill host directly by destruction of vital tissue
Radiation sensitivity	Radiation sensitivity near that of normal parent cell; rarely treated with radiation	Radiation sensitivity increased in rough proportion to malignancy; often treated with radiation
Behavior in tissue	Cells cohesive and inhibited by mutual contact	Cells do not cohere; frequently not inhibited by mutual contact
Resemblance to tissue of origin	Cells and architecture resemble tissue of origin	Cells atypical and pleomorphic; disorganized bizarre architecture
Mitotic figures	Mitotic figures rare and normal	Mitotic figures may be numerous and abnormal in polarity and configuration
Shape of nucleus	Normal and regular; show usual stain affinity	Irregular; nucleus frequently hyperchromatic
Size of nucleus	Normal; ratio of nucleus to cytoplasm near normal	Frequently large; nucleus to cytoplasm ratio increased
Nucleolus	Not conspicuous	Hyperchromatic and larger than normal

Source: Maronpot, R.R., *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, 1991. With permission.

TABLE 14.22
Selected Taxonomy of Neoplasia

Tissue	Benign Neoplasia^a	Malignant Neoplasia^b
Epithelium		
Squamous	Squamous cell papilloma	Squamous cell carcinoma
Transitional	Transitional cell papilloma	Transitional cell carcinoma
Glandular		
Liver cell	Hepatocellular adenoma	Hepatocellular carcinoma
Islet cell	Islet cell adenoma	Islet cell adenocarcinoma
Connective tissue		
Adult fibrous	Fibroma	Fibrosarcoma
Embryonic fibrous	Myxoma	Myxosarcoma
Cartilage	Chondroma	Chondrosarcoma
Bone	Osteoma	Osteosarcoma
Fat	Lipoma	Liposarcoma
Muscle		
Smooth muscle	Leiomyoma	Leiomyosarcoma
Skeletal muscle	Rhabdomyoma	Rhabdomyosarcoma
Cardiac muscle	Rhabdomyoma	Rhabdomyosarcoma
Endothelium		
Lymph vessels	Lymphangioma	Lymphangiosarcoma
Blood vessels	Hemangioma	Hemangiosarcoma
Lymphoreticular		
Thymus	(Not recognized)	Thymoma
Lymph nodes	(Not recognized)	Lymphosarcoma (malignant lymphoma)
Hematopoietic		
Bone marrow	(Not recognized)	Leukemia
		Granulocytic
		Monocytic
		Erythroleukemia
Neural tissue		
Nerve sheath	Neurilemmoma	Neurogenic sarcoma
Glioma	Glioma	Malignant glioma
Astrocytes	Astrocytoma	Malignant astrocytoma
Embryonic cells	(Not recognized)	Neuroblastoma

Source: Maronpot, R.R., *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, 1991. With permission.

^a “-oma,” benign neoplasm.

^b “Sarcoma,” malignant neoplasm of mesenchymal origin; “carcinoma,” malignant neoplasm of epithelial origin.

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REFERENCES

1. Maronpot, R. R., Chemical carcinogenesis, in *Handbook of Toxicologic Pathology*, Haschek, W. M. and Rousseaux, C. G., Eds., Academic Press, San Diego, CA, 1991, chap. 7.
2. Pitot, H. C. and Dragon, Y. P., Stage of tumor progression, progressor agents, and human risk, *Proc. Soc. Exp. Biol. Med.*, 202, 37, 1993.
3. Pitot, H. C., Endogenous carcinogenesis: The role of tumor promotion, *Proc. Soc. Exp. Biol. Med.*, 198, 661, 1991.
4. Butterworth, B. E. and Goldsworthy, T. L., The role of cell proliferation in multistage carcinogenesis, *Proc. Soc. Exp. Biol. Med.*, 198, 683, 1991.
5. Haseman, J. K., Huff, J. E., Rao, G. N., and Eustis, S. L., Sources of variability in rodent carcinogenicity studies, *Fundam. Appl. Toxicol.*, 12, 793, 1989.
6. Wilbourn, J., Haroun, L., Haseltine, E., Kaldor, J., Partensky, C., and Vainio, H., Response of experimental animals to human carcinogens: An analysis based upon IARC monographs program, *Carcinogenesis*, 7, 1853, 1986, cited in Ecobichon.⁴⁰
7. Robinson, D., The International Life Sciences Institute role in the evaluation of alternative methodologies for the assessment of carcinogenic risk, *Toxicol. Pathol.*, 26, 474, 1998.

8. Boverhoff, D. R., Chamberlain, M. P., Elcombe, C. R., Gonzalez, F. J., and Heflich, R. H., Transgenic animal models in toxicology: Historical perspective and future outlook, *Toxicol. Sci.*, 121, 233, 2011.
9. Gulezian, D., Jacobson-Kram, D., McCullough, B., Olson, H., Recio, L., Robinson, D., Storer, R., Tennant, R., Ward, J. M., and Neumann, D., Use of transgenic animals for carcinogenicity testing: Considerations and implications for risk assessment, *Toxicol. Pathol.*, 28, 482, 2000.
10. ICH Harmonized Tripartite Guideline S1B. *Testing for Carcinogenicity of Pharmaceuticals*, July 1997.
11. Pritchard, J. B., French, J. E., Davis, B. J., and Haseman, J. K., The role of transgenic mouse models in carcinogen identification, *Environ. Health Perspect.*, 111, 454, 2003.
12. Wells, M. Y. and Williams, E. S., The transgenic mouse assay as an alternative test method for regulatory carcinogenicity studies—Implications for REACH, *Regul. Toxicol. Pharmacol.*, 53, 150, 2009.
13. Alden, C. L., Lynn, C., Bourdeau, A., Morton, D., Sistare, F. D., Kadambi, V. J., and Silverman, L., A critical review of the effectiveness of rodent pharmaceutical carcinogenesis testing in predicting for human risk, *Vet. Pathol.*, 48, 784, 2011.
14. Pintadosi, S. and Sullivan, J. B., Chemical and environmental carcinogenesis, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R., Eds., Williams & Wilkins, Baltimore, MD, 1992, chap. 8.
15. Maynard, R. L., Cameron, K. M., Fielder, R., McDonald, A., and Wadge, A., Setting air quality standards for carcinogens: An alternative to mathematical quantitative risk assessment—discussion paper, *Human Exp. Toxicol.*, 14, 175, 1995.
16. Weisburger, J. H. and Williams, G. M., Chemical carcinogens, in *Cassarett and Doull's Toxicology: The Basic Science of Poisons*, 2nd edn., Doull, J., Klaassen, C. D., and Amdur, M. O., Eds., Macmillan, New York, 1980, chap. 6.
17. Pitot, H. C., The dynamics of carcinogenesis: Implications for human risk, C.I.T. Activities, *Chem. Ind. Inst. Toxicol.*, 13(6), 1993.
18. Gad, S. C. and Weil, C. S., *Statistics and Experimental Design for Toxicologists*, Telford Press, Caldwell, NJ, 1986.
19. Chu, K., *Percent Spontaneous Primary Tumors in Untreated Species Used at NCI for Carcinogen Bioassays*, NCI Clearing House, 1977, cited in Gad and Weil.¹⁸
20. Fears, T. R., Tarone, R. E., and Chu, K. C., False positive and false negative rates for carcinogenicity screens, *Cancer Res.*, 27, 1941, 1977, cited in Gad and Weil.¹⁸
21. Page, N. P., Concepts of a bioassay program in environmental carcinogenesis, in *Environmental Cancer*, Kraybill, H. F. and Mehlman, M. A., Eds., Hemisphere, New York, 1977, pp. 87–171, cited in Gad and Weil.¹⁸
22. Gart, J. J., Chu, K. C., and Tarone, R. E., Statistical issues in interpretation of chronic bioassay tests for carcinogenicity, *J. Natl. Cancer Inst.*, 62, 957, 1979, cited in Gad and Weil.¹⁸
23. Tarone, R. E., Chu, K. C., and Ward, J. M., Variability in the rates of some common naturally occurring tumors in Fischer 344 rats and (C57BL/6NXC3H/HEN) F' (B6C3F₁) mice, *J. Natl. Cancer Inst.*, 66, 1175, 1981, cited in Gad and Weil.¹⁸
24. Rao, G. N., Haseman, J. K., Grumbein, S., Crawford, D. D., and Eustis, S. L., Growth, body weight, survival and tumor trends in (C57BL/6 × C3H/HeN)F₁ (B6C3F₁) mice during a nine year period, *Toxicol. Pathol.*, 18, 71, 1990.
25. Lang, P. L., *Spontaneous Neoplastic Lesions in the Crl:CDI® (ICR) BR Mouse*, Charles River Laboratories, Wilmington, MA, 1987.
26. Goodman, D. G., Ward, J. M., Squire, R. A., Chu, K. C., and Linhart, M. S., Neoplastic and nonneoplastic lesions in aging F344 rats, *Toxicol. Appl. Pharmacol.*, 48, 237, 1979, cited in Gad and Weil.¹⁸
27. Bomhard, E., Karbe, E., and Loeser, E., Spontaneous tumors of 2000 Wistar TNO/W.70 rats in two year carcinogenicity studies, *J. Environ. Pathol. Toxicol. Oncol.*, 7, 35, 1986.
28. Walsh, K. M. and Poteracki, J., Spontaneous neoplasms in control Wistar rats, *Fundam. Appl. Toxicol.*, 22, 65, 1994.
29. Haseman, J. K., Patterns of tumor incidence in two year cancer bioassay feeding studies in Fischer 344 rats, *Fundam. Appl. Toxicol.*, 3, 1, 1983.
30. Rao, G. N., Haseman, J. K., Grumbein, S., Crawford, D. D., and Eustis, S. L., Growth, body weight, survival, and tumor trends in F344/N rats during an eleven year period, *Toxicol. Pathol.*, 18, 61, 1990.
31. Poteracki, J. and Walsh, K. M., Spontaneous neoplasms in control Wistar rats: A comparison of reviews, *Toxicol. Sci.*, 45, 1, 1998.
32. Linkov, I., Wilson, R., and Gray, G. M., Anticarcinogenic responses in rodent bioassays are not explained by random effects, *Toxicol. Sci.*, 43, 1, 1998.
33. United States Environmental Protection Agency Health Effects Testing Guidelines, Code of Federal Regulations, 40, part 798, 1989.
34. Young, J. K., Hall, R. L., O'Brien, P., Strauss, V., and Vahle, J. L., Best practices for clinical pathology testing in carcinogenicity studies. STP/ASCP Clinical Pathology in Carcinogenicity Working Group, *Toxicol. Pathol.*, 39, 429, 2011.
35. Huff, J., Cirvello, J., Haseman, J., and Bucher, J., Chemicals associated with site-specific neoplasia in 1394 long term carcinogenesis experiments in laboratory rodents, *Environ. Health Perspect.*, 93, 247, 1991.
36. Gold, L. S., Slone, T. H., Manley, N. B., and Bernstein, L., Target organs in chronic bioassays of 533 chemical carcinogens, *Environ. Health Perspect.*, 93, 233, 1991.
37. Alison, R. H., Capen, C. C., and Prentice, D. E., Neoplastic lesions of questionable significance to humans, *Toxicol. Pathol.*, 22, 179, 1994.
38. Williams, G. M. and Iatropoulos, M. J., Principles of testing for carcinogenic activity, in *Principles and Methods of Toxicology*, 4th edn., Hayes, A. W., Ed., Taylor & Francis, Philadelphia, PA, 2001, chap. 20.
39. Ashby, J., Doerr, N. G., Flamm, F. G., Harris, J. E., Hughes, D. H., Johannsen, F. R., Lewis, S. C. et al., A scheme for classifying carcinogens, *Regul. Toxicol. Pharmacol.*, 12, 270, 1990.
40. Ecobichon, D. J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, chap. 6.
41. Yager, J. P., Zurlo, J., and Ni, N., Sex hormones and tumor promotion in liver, *Proc. Soc. Exp. Biol. Med.*, 198, 667, 1991.
42. Tennant, R. W., French, J. E., and Spalding, J. W., Identifying chemical carcinogens and assessing potential risk in short term bioassays using transgenic mouse models, *Environ. Health Perspect.*, 103, 942, 1995.
43. Yamamoto, S., Hayashi, Y., Misumori, K., and Nomura, T., Rapid carcinogenicity testing system with transgenic mice harboring human prototype c-HRAS gene, *Lab Anim. Sci.*, 47, 121, 1997.

15 Histopathology

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GENERAL INFORMATION

(TABLES 15.1 THROUGH 15.5)

TABLE 15.1
Solutions Commonly Used for Tissue Fixation

Fixative Solution	Primary Chemical(s)	Use	Tissue Recommended (Time)	Advantages/Disadvantages	Comments
Formalin saline (10%)	Formaldehyde (37%–40% gas in water) Sodium chloride	General fixative for light microscopy	All (1–3 days) CNS (1–4 weeks)	Easily used, rapid penetration, formic acid formation	Specimen <5 mm thickness, volume of tissue to fixative 1:>10 Move to 70% alcohol
Neutral buffered formalin (10%)	Formaldehyde (37%) Sodium phosphate buffers	General fixative for light microscopy	All (1–7 days) CNS (1–4 weeks)	Easily used, rapid penetration, longer storage without formic acid formation	Specimen <5 mm thickness, volume of tissue to fixative 1:>10 Move to 70% alcohol
Formalin ammonium bromide	15% Formalin Ammonium bromide	Special fixative for nervous system	CNS (3–30 days)	Preferred for Cajal gold stain for astrocytes	
Bouin's	Picric acid Formaldehyde (37%) Acetic acid (15:5:1)	Brilliant colors, sharp nuclear details	Ovaries, testis, thyroid, adrenal (4–18 h)	Rapid fixation, must be replaced with 70% alcohol	Specimens thin, collect within minutes of death
Zenker's	Mercuric chloride Potassium dichromate Sodium sulfate	Brilliant colors, sharp nuclear details	Eye (6–18 h)	Rapid fixation, less shrinkage, poor penetration	Collect within minutes of death
Zenker's acetic formalin	Zenker's base Formaldehyde (37%) Acetic acid	Brilliant colors, sharp nuclear details	Bone with marrow (6–18 h)	Good for hematopoietic tissues	Specimens thin
Carnoy's	Absolute alcohol Chloroform Acetic acid	Sharp nuclear details	All (1–3 h)	Rapid penetration, quick fixation, lysis of erythrocytes	Small specimens Transfer to alcohol
Helley's (Zenker's formalin)	Zenker's base Formaldehyde (37%)	Brilliant colors, sharp nuclear details	Bone marrow (6–18 h)	Good for hematopoietic tissues	Specimens thin
Glutaraldehyde (2%–6%)	Glutaraldehyde (25%) Phosphate buffers	General fixative for electron microscopy	All (6–24 h)	Rapid fixation, used for perfusion fixation of deep tissues, example, CNS	Secondary fixation: osmium tetroxide, specimens <1 mm ³ , collect within minutes of death or perfuse
Karnovsky's	Paraformaldehyde Glutaraldehyde (50%) Phosphate buffers	General fixative for electron microscopy	All (1–2 h)	Rapid fixation, used for perfusion fixation of deep tissues, example, CNS	Specimens <1 mm ³ collect within minutes of death or perfuse, keep fixative 4°C, store in sucrose buffer

Sources: Sanders, B.J., Animal Histology Procedures of the Pathological Technology Section of the National Cancer Institute, HEW Publication No. (NIH) 72275 Superintendent of Documents, U.S. Government Printing Office, Washington, DC, 1972; Luna, L.G., Ed., *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd edition, McGraw-Hill, New York, 1968, chap. 1; Luna, L.G., *Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts*, American Histolabs, Inc., Gaithersburg, MD, 1992.

TABLE 15.2
Stains Commonly Used in Histopathology

Procedure	Dye(s)	Use or Structures Stained	Result
Hematoxylin and eosin (H&E)	Hematoxylin, eosin, or phloxine–eosin	General light microscopy	Nucleus: blue Cytoplasm: red Red blood cell: red
Masson trichrome	Iron hematoxylin, acid fuchsin, Ponceau 2R, light green	General light microscopy	Nucleus: black Cytoplasm: red Collagen: green Reticular fibers: green
Mallory's phosphotungstic acid hematoxylin (PTAH)	Hematoxylin and phosphotungstic acid	General light microscopy	Nucleus: blue Muscle fibers: blue Collagen: red Fibrin: blue
Verhoeff–Van Gieson	Alcoholic hematoxylin, ferric chloride, and Verhoeff iodine	Elastic fibers	Elastic fibers: black Collagen: red Nucleus: blue Background: yellow
Weigert's elastic	Resorcin–basic fuchsin, iron hematoxylin, and Van Gieson's solution (acid fuchsin, picric acid)	Elastic fibers	Nucleus: blue Cytoplasm: yellow Collagen: red Elastic fibers: black
Gomori's reticulum	Ammoniacal silver, ferric ammonia sulfate, and gold chloride	Reticulum fibers	Reticulum fibers: black Background: gray
Wilder's reticulum	Ammoniacal silver, gold chloride, and Mayer's hematoxylin	Reticulum fibers	Reticulum fibers: black Collagen: red Nucleus: blue
McManus periodic acid–Schiff (PAS) with and without diastase digestion	PAS's reagent, light green or hematoxylin, either Harris's or Mayer's	Carbohydrates: glycogen, glycoproteins, glycolipids in cytoplasm, basement membrane, or capsule of fungi	Glycogen: rose to purple Mucin: blue Basement membrane: pink
PAS–Alcian blue	PAS's reagent Alcian blue, pH 2.5 or pH 1.0	Acid mucopolysaccharides, glycosaminoglycans in mucus and cartilage	Polysaccharides: red Mucosubstances: red Acid mucopolysaccharides: blue
PAS–hematoxylin	PAS's reagent, Harris hematoxylin, and light green	Carbohydrates: glycogen, glycoproteins, glycolipids in cytoplasm, basement membrane, or capsule of fungi	Glycogen: rose to purple Mucin: blue Basement membrane: pink
PAS–MS	PAS's reagent, methenamine silver	Reticulum fibers	Reticular fibers: blue
Mowry's colloidal iron	Ferric chloride, colloidal iron, acetic acid, and ferrocyanide	Acid mucopolysaccharides in cytoplasm and mucins	Hyaluronic acid: blue Sialomucin: blue
Armed Forces Institute of Pathology: mucosubstances	Aldehyde fuchsin Mucicarmine and Mayer's hematoxylin	Mucosubstances in mast cells and mucins Mucosubstances in mucin	Mast cells, hyaluronic acid, sialomucin, sulfated mucosubstances
Mayer's mucicarmine			Mucin: red Nucleus: blue
May–Grunwald–Giemsa	Giemsa	Polychromasia	Mast cells: blue Nucleus: blue Cytoplasm: red to blue
Nocht azure–eosin	Azure A, eosin B	Polychromasia	Nucleus: blue Secretory granules: red Bacteria: blue Mast cells: blue
Tomlinson–Grocott	Toluidine blue	Polychromasia	Nucleus: blue Cytoplasm: red to blue Mast cells: dark blue
Lipid (requires frozen sections and fixation without lipid solvents)	Sudan black B	Neutral lipids and triglycerides in cytoplasmic fat	Compound lipids: blue black

(continued)

TABLE 15.2 (continued)
Stains Commonly Used in Histopathology

Procedure	Dye(s)	Use or Structures Stained	Result
Lipid (requires frozen sections and fixation without lipid solvents)	Sudan IV or Sudan red	Neutral lipids and triglycerides in cytoplasmic fat	Neutral lipids: red
Armed Forces Institute of Pathology: fat (requires frozen sections and fixation without lipid solvents)	Oil red O	Neutral lipids and triglycerides in cytoplasmic fat	Simple neutral lipids: red
Bennhold	Congo red and Mayer's hematoxylin	Amyloid	Amyloid: pink to red Nucleus: blue
Lillie's amyloid	Crystal violet	Amyloid	Amyloid: red purple Background: blue purple
Feulgen's	HCl and Schiff's reagent	Deoxyribonucleic acid (DNA) in nuclei	Nuclear DNA: red
Ribonucleic acid (RNA) (with and without ribonuclease)	Methylene blue or toluidine blue	RNA	RNA: blue staining (which is lost after pretreatment with ribonuclease)
Kluver-Barrera	Luxol fast blue and cresyl violet	Nervous tissue: nerve fibers	Myelin: greenish blue Cells pink to violet
Einarson	Gallocyanin	Nervous tissue: neurons	Nissl substance: dark blue Cytoplasm: pale blue
Bodian silver	Protargol and hydroquinone	Nervous tissue: argyrophilic granules and nerve fibers	Argyrophilic granules: black Nerve fibers: black Nucleus: black Background: gray
Gridley's fungus	Chromic acid, Feulgen's reagent, aldehyde fuchsin, and metanil yellow	Fungi	Fungi: deep purple Elastic tissue: purple Mucin: purple Background: yellow
Grocott's fungus	Methenamine silver, nitrate, gold chloride, and light green	Fungi	Fungi: black Mucin: gray Background: pale green
Brown and Brenn Gram	Crystal violet, Gram's iodine solution, and basic fuchsin	Bacteria: bacterial wall	Bacteria: Gram positive: blue Gram negative: red Nucleus: red Background: yellow
Ziehl-Neelsen acid fast	Carbol fuchsin, acid alcohol, and methylene blue	Acid-fast bacteria and acid-fast pigment	Acid-fast bacteria: red Acid-fast pigment: red Nucleus: blue
Von Kossa's calcium	Silver nitrate, sodium thiosulfate (hypo), and nuclear fast red	Calcium salts and bone	Calcium salts: black Nucleus: red Cytoplasm: light pink
Phosphate (acid fixatives cannot be used when identifying calcium phosphate)	Silver nitrate and hydroquinone	Bone, ossification: calcification	Calcium phosphate: black
Perl's iron	Potassium ferrocyanide, HCL, and nuclear fast red	Iron (ferric ions)	Iron (ferric ions): dark blue precipitate Nucleus: red
Lead citrate	Lead citrate	Electron microscopy	Organelle ultrastructure detailed by electron-dense deposits
Uranium acetate	Uranium acetate	Electron microscopy	Organelle ultrastructure detailed by electron-dense deposits
Janigan	Thioflavine T	Fluorescence microscopy: juxtaglomerular cells	Juxtaglomerular cell granules: golden yellow
Vassar-Culling	Thioflavine T	Fluorescence microscopy: amyloid	Amyloid: white or yellow

TABLE 15.2 (continued)
Stains Commonly Used in Histopathology

Procedure	Dye(s)	Use or Structures Stained	Result
Acridine orange fluorescence	Acridine orange	Fluorescence microscopy: fungi, nucleic acids, and virus particles	Fungi: bright orange DNA: yellowish green RNA: reddish orange
Immunofluorescence	Antibodies coupled to fluorescent dyes, such as fluorescein isothiocyanate or rhodamine	Fluorescence microscopy: various antigenic structures	Positive antigen–antibody fluorescence
Histochemistry: acid phosphatase	Glycerophosphate, lead nitrate, ammonium sulfide	Light and electron microscopy	Lysosomes: black
Histochemistry: dehydrogenases	Monotetrazole	Light and electron microscopy	Mitochondria: dark dense precipitate
Histochemistry: peroxidases	3,3' Diaminoazobenzidine	Light and electron microscopy	Peroxidase sites: dense precipitate
Immunohistochemistry: peroxidase	Peroxidase bound to antibody, 3,3' Diaminoazobenzidine	Light and electron microscopy	Antigen sites: dense precipitate
Immunohistochemistry: ferritin	Ferritin bound to antibody	Light and electron microscopy	Antigen sites: dense precipitate
Avidin–biotin–enzyme complexes	Biotinylated antibody, avidin–enzyme complex chromagen	Light microscopy	Antigen sites: dense or colored precipitate

Sources: Sanders, B.J., Animal Histology Procedures of the Pathological Technology Section of the National Cancer Institute, HEW Publication No. (NIH) 72275 Superintendent of Documents, U.S. Government Printing Office, Washington, DC, 1972; Luna, L.G., Ed., *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd edition, McGraw-Hill, New York, 1968, chap. 1; Luna, L.G., *Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts*, American Histolabs, Inc., Gaithersburg, MD, 1992; Sheehan, D.C. and Hrapchak, B.B., *Theory and Practice of Histotechnology*, 2nd edition, C. V. Mosby, Saint Louis, MO, 1980.

TABLE 15.3
Common Abbreviations and Codes Used in Histopathology

Code	Finding or Observation
+	Minimal grade lesion
++ (2)	Mild or slight grade lesion
+++ (3)	Moderate grade lesion
++++ (4)	Marked or severe grade lesion
+++++ (5)	Very severe or massive grade lesion
(No Entry)	Lesion not present or organ/tissue not examined
+	Tissue examined microscopically
–	Organ/tissue present, no lesion in section
A	Autolysis precludes examination
B	Primary benign tumor
I	Incomplete section of organ/tissue or insufficient tissue for evaluation
M	Primary malignant tumor
M	Organ/tissue missing, not present in section
N	No section of organ/tissue
N	Normal, organ/tissue within normal limits
NCL	No corresponding lesion for gross finding
NE	Organ/tissue not examined
NRL	No remarkable lesion, organ/tissue within normal limits
NSL	No significant lesion, organ/tissue within normal limits
P	Lesion present, not graded (e.g., cyst, anomaly)
R	Recut of section with organ/tissue
U	Unremarkable organ/tissue within normal limits
WNL	Organ/tissues within normal limits
X	Not remarkable organ/tissue, normal
X	Incidence of listed morphology, lesion present

TABLE 15.4
Frequently Used Grading Schemes for Histopathology^a

A1	A2	B	C	D	E
(1) Minimal <1%–25%	(1) Minimal <1%–15%	(1) Minimal <1%	(1) Slight 1%–25%	(1) Minimal <1%	1 = 1–4 foci
(2) Mild 26%–50%	(2) Mild 16%–35%	(2) Slight 1%–25%	(2) Mild 26%–50%	(2) Mild 1%–30%	2 = 5–8 foci
(3) Moderate 51%–75%	(3) Moderate 36%–60%	(3) Moderate 26%–50%	(3) Moderate 51%–75%	(3) Moderate 31%–60%	3 = 9–12 foci
(4) Marked 76%–100%	(4) Marked 61%–100%	(4) Moderately severe 51%–75%	(4) Severe 76%–100%	(4) Severe 61%–90%	4 = >12 foci
		(5) Severe 76%–100%		(5) Very severe or massive 91%–100%	

Sources: Adapted from Hardisty, J.F. and Eustis, S.L., Toxicological pathology: A critical stage in study interpretation, in *Progress in Predictive Toxicology*, Clayson, D.B., Munro, I.C., Shubik, P., and Swenberg, J.A., Eds., Elsevier, New York, 1990, p. 41; World Health Organization, *Principles and Methods for Evaluating the Toxicity of Chemicals. Part 1*, Environmental Health Criteria 6, World Health Organization, Geneva, Switzerland, 1978, p. 192.

Note: The relative proportion of an affected organ associated with specific severity term: *Minimal* is a very small amount, *Slight* is a very small to small amount, *Mild* is a small amount, *Moderate* is a middle or median amount, *Marked* is a large amount, *Moderately severe* is also a large amount, *Severe* is a very large amount, and *Very severe or massive* is also a very large amount.

^a A1 and A2 are examples of four-severity grade schemes commonly used by pathologists in the National Toxicology Program; B and D are examples of five-severity grade schemes that have been used by other researchers, often for pharmaceutical companies; C is another four-severity grade scheme similar to A1 using different terminology; and E is an example for grading of a quantifiable lesion.

TABLE 15.5
Goals for Protocol Tissue Availability during Histopathology^a

Organ or Lesion	Fischer 344 Rat ^{b,c}		B6C3F ₁ Mouse ^d	
	Fair Range (%) >Fair = Good	Historical Availability	Fair Range (%) >Fair = Good	Historical Availability
Gross lesions	<100	Unknown	<100	Unknown
Adrenal cortex	96–97.9	98.7 (m) ^b 98.7 (f) ^b	92–95.9	97.0 (m) 98.6 (f)
Adrenal medulla	96–97.9	97.0 (m) ^b 96.1 (f) ^b	92–95.9	95.9 (m) 96.9 (f)
Bone with marrow	96–97.9	97.1 (m) ^b 97.1 (f) ^b	92–95.9	99.2 (m) 99.4 (f)
Brain	96–97.9	99.7 (m) ^b 99.8 (f) ^b	96–97.9	99.7 (m) 99.5 (f)
Clitoral gland	92–95.9	90.3 (f) ^b		100.0 (f)
Epididymis	92–95.9	98.3 (m) ^b	88–93.9	98.9 (m)

TABLE 15.5 (continued)
Goals for Protocol Tissue Availability during Histopathology^a

Organ or Lesion	Fischer 344 Rat ^{b,c}		B6C3F ₁ Mouse ^d	
	Fair Range (%) >Fair = Good	Historical Availability	Fair Range (%) >Fair = Good	Historical Availability
Esophagus	92–95.9	95.6 (m) ^c 96.3 (f) ^c	88–93.9	
Gallbladder			84–91.9	87.3 (m) 90.2 (f)
Heart	96–97.9	99.9 (m) ^b 99.9 (f) ^b	96–97.9	99.8 (m) 99.8 (f)
Intestine, large		95.6 (m) ^c 96.8 (f) ^c		
Cecum	88–93.9		88–93.9	
Colon	88–93.9		88–93.9	
Rectum	88–93.9		88–93.9	
Intestine, small		96.3 (m) ^c 97.8 (f) ^c		
Duodenum	88–93.9		88–93.9	
Ileum	88–93.9		88–93.9	
Jejunum	88–93.9		88–93.9	
Islets, pancreas	92–95.9	98.5 (m) ^b 99.0 (f) ^b	88–93.9	97.7 (m) 97.5 (f)
Kidney	96–97.9	99.7 (m) ^b 99.7 (f) ^b	96–97.9	99.6 (m) 99.4 (f)
Larynx	92–95.9		88–93.9	
Liver	96–97.9	99.8 (m) ^b 99.9 (f) ^b	96–97.9	99.7 (m) 99.7 (f)
Lung	96–97.9	99.8 (m) ^b 99.9 (f) ^b	96–97.9	99.7 (m) 99.8 (f)
Lymph node	92–95.9		88–93.9	
Nasal cavity	96–97.9	97.6 (m) ^b 96.8 (f) ^b	96–97.9	98.1 (m) 98.4 (f)
Ovary	96–97.9	99.6 (f) ^b	92–95.9	97.0 (f)
Pancreas	92–95.9	98.8 (m) ^b 99.2 (f) ^b	88–93.9	98.3 (m) 98.3 (f)
Parathyroid	60–79.9	89.4 (m) ^b 88.0 (f) ^b	50–69.9	76.9 (m) 77.9 (f)
Pituitary gland	92–95.9	98.3 (m) ^b 99.0 (f) ^b	84–91.9	91.1 (m) 94.5 (f)
Preputial gland	92–95.9	95.4 (m) ^b		100.0 (m)
Prostate	92–95.9	98.6 (m) ^b	88–93.9	97.5 (m)
Salivary gland	96–97.9	97.9 (m) ^b 97.4 (f) ^b	92–95.9	99.3 (m) 98.5 (f)
Seminal vesicle	92–95.9		88–93.9	
Skin	96–97.9		96–97.9	
Spleen	96–97.9	99.5 (m) ^b 99.7 (f) ^b	92–95.9	98.9 (m) 98.9 (f)

(continued)

TABLE 15.5 (continued)
Goals for Protocol Tissue Availability during Histopathology^a

Organ or Lesion	Fischer 344 Rat ^{b,c}		B6C3F ₁ Mouse ^d	
	Fair Range (%) >Fair = Good	Historical Availability	Fair Range (%) >Fair = Good	Historical Availability
Forestomach and stomach	92–95.9	98.8 (m) ^c 98.6 (f) ^c	92–95.9	
Testis	96–97.9	99.6 (m) ^b	92–95.9	99.4 (m)
Thymus		84.9 (m) ^b		77.1 (m)
Chronic	60–79.9	87.5 (f) ^b	50–69.9	86.6 (f)
Subchronic	96–97.9		92–95.9	
Thyroid	94–97.9	98.9 (m) ^b 99.4 (f) ^b	92–95.9	98.5 (m) 98.5 (f)
Trachea	92–95.9	94.7 (m) ^c 95.6 (f) ^c	88–93.9	
Urinary bladder	92–95.9	98.0 (m) ^b 98.4 (f) ^b	92–95.9	97.6 (m) 96.7 (f)
Uterus	92–95.9	99.1 (f) ^c	92–95.9	

Sources: Hardisty, J.F. and Boorman, G.A., National Toxicology Program pathology quality assurance procedures, in *Managing Conduct and Data Quality of Toxicology Studies*, Hoover, K.B., Baldwin, J.K., Velner, A.F., Whitmire, C.E., Davies, C.L., and Bristol, D.W., Eds., Princeton Scientific, Princeton, NJ, 1986, p. 263; Haseman, J.K., Arnold, J., and Eustis, S.L., Tumor incidences in Fischer 344 rats: NTP historical data, in *Pathology of the Fischer Rat*, Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery, C.A., and MacKenzie, W.F., Eds., Academic Press, New York, 1990, p. 555.

^a The goal that 100% of all protocol organs and lesions will be available for histopathology is rarely possible because of sampling errors, lost tissues, and very small organs.

^b 7142 rats: 3572 males (m), 3570 females (f).

^c 3919 rats: 1936 males (m), 1983 females (f).

^d 7596 mice: 3807 males (m), 3789 females (f).

COMMON MICROSCOPIC POSTMORTEM CHANGES IN LABORATORY RATS⁹

Adrenal gland, cortex

Condensation of cells of zona glomerulosa (40 min*)

Karyoklasis (clumping of chromatin) in epithelial cells of zona fasciculata and zona reticularis (8 h)

Adrenal gland, medulla

Nuclear condensation and pyknosis (2 h)

Increased cytoplasmic vacuolation (2 h)

Ampullary gland

Sloughed and disruption of epithelium (<20 min)

Blood

Hemolysis and pigmentation of adjacent tissues (>16 h)

Blood vessel (major)

Aorta—formation of clear and pale staining areas between elastic laminae (<20 min)

Aorta—darkening and condensation of smooth muscle nuclei (16 h)

Bone (sternum)

Bone and cartilage—no remarkable postmortem changes at 16 h

Bone marrow

Pyknosis of megakaryocytes (2 h)

Brain

Increased clear space around oligodendroglia in white matter (2 h)

Formation of clear space around cortical astrocytes (4 h)

Increased numbers of shrunken hyperchromatic neurons (8 h)

Pyknosis of oligodendroglia in corpus callosum (8 h)

* Time of onset after death.

- Pyknosis of cells in granular layer of the cerebellum (12 h)
Increased foamy and vacuolated cytoplasm of cortical neurons (12 h)
- Brown fat (perithymic)
Granular cytoplasm (4 h)
Blood-filled vessels (8 h)
Condensed nuclei (8 h)
Formation of intracellular soap, basophilic deposits (8 h)
- Bulbourethral gland
Increased granularity and lack of uniformity to the epithelial cell cytoplasm (12 h)
- Clitoral gland
No remarkable postmortem changes at 16 h
- Coagulating gland
Pyknosis of villous epithelium (20 min)
Pyknosis of basal epithelium (20 min)
Disruption of epithelium (4 h)
- Ductus (vas) deferens
Formation of intercellular clear clefts between adjacent epithelial cells (20 min)
- Epididymis
Formation of intercellular clear clefts between adjacent epithelial cells (16 h)
- Esophagus
Pale cytoplasm of polyhedral epithelial cells (40 min)
Formation of interfiber clear spaces in muscle (40 min)
Formation of vesicular spaces within collagen of the submucosa (8 h)
Pyknosis of fibrocyte nuclei in submucosa (8 h)
Absence of mitotic figures in basal epithelium layer (12 h)
- Eye
Shrinkage of nuclei and formation of clear spaces and clefts in the substantia propria of cornea (20 min)
Absence of mitotic figures in anterior epithelium of the cornea (40 min)
Condensation of nuclei in the retina (8 h)
- Fat
No remarkable postmortem changes at 16 h
- Fat, brown (*see* Brown Fat)
- Harderian gland
Pyknosis of glandular epithelial cells (4 h)
Karyorrhexis of glandular epithelial cells (12 h)
- Heart
Condensation and darkening of endothelial nuclei (20 min)
Formation of interfiber clear spaces in muscle (40 min)
- Intestine, large—cecum
Loss of superficial epithelial cells (<20 min)
Saprophytic bacilli in the lamina propria (<20 min)
Formation of interfiber clear spaces and pyknosis of myofiber nuclei (1 h)
- Intestine, large—colon
Loss of superficial epithelial cells (<20 min)
Saprophytic bacilli in the lamina propria (<20 min)
Formation of interfiber clear spaces and pyknosis of myofiber nuclei (40 min)
Disruption and sloughing of crypt epithelium (2 h)
Loss of crypt epithelial cells (4 h)
Basophilia of the lamina propria caused by the presence of large numbers of bacilli (4 h)
Pyknosis of remaining epithelial cells (8 h)
Loss of cells in lamina propria (12 h)
- Intestine, small—duodenum
Loss of villar epithelium (<20 min)
Formation of interfiber clear spaces and pyknosis of myofiber nuclei (40 min)
Loss of cells in the villous lamina propria (4 h)
Lateral separations in crypt epithelium (4 h)
Loss of epithelium from crypts (12 h)
Loss of cells in basal lamina propria (12 h)
- Intestine, small—ileum
Loss of villar epithelium (<20 min)
Formation of interfiber clear spaces and pyknosis of myofiber nuclei (40 min)
Lateral separations in crypt epithelium (40 min)
Loss of cells in the villous lamina propria (1 h)
Loss of cells in basal lamina propria (2 h)
Pyknosis of Peyer's patch lymphocytes (4 h)
Loss of epithelium from crypts (12 h)
Total loss of mucosal epithelium (12 h)
- Intestine, small—jejunum
Loss of villar epithelium (<20 min)
Loss of cells in the villous lamina propria (<20 min)
Formation of interfiber clear spaces and pyknosis of myofiber nuclei (40 min)
Lateral separations in crypt epithelium (1 h)
Loss of cells in basal lamina propria (12 h)
Loss of epithelium from crypts (>16 h)
- Islets, pancreatic
Pyknosis of peripheral islet cells (4 h)
Pyknosis of most of islet cells (16 h)
- Kidney
Pyknosis and vacuolated cytoplasm of distal convoluted tubular epithelium (40 min)
Pyknosis of majority of nuclei of ascending and descending tubules of loops of Henle at the corticomedullary junction (2 h)
Contraction of nuclei and increased granularity of cytoplasm of proximal convoluted tubular epithelium (4 h)

- Separation and sloughing of pelvic urothelium (4 h)
- Majority of glomerular cell nuclei pyknotic (8 h)
- Formation of clear space around the loops of Henle deep in the medulla (8 h)
- Pyknotic nuclei in the collecting tubule epithelium of the cortex (12 h)
- Lacrimal gland (exorbital)
 - Nuclear condensation of acinar epithelium (12 h)
- Liver
 - Sinusoidal accumulation of blood (resembles congestion) (<20 min)
 - Presence of saprophytic bacilli (2 h)
- Lung (immersion-fixed)
 - Parenchymal blood accumulation (resembles congestion) (<20 min)
 - Separation of bronchiolar epithelium from the lamina propria (<20 min)
 - Presence of proteinaceous fluid in alveolar spaces (40 min)
 - Pyknosis of a majority of alveolar cells (4 h)
 - Pyknosis and sloughing of bronchiolar epithelium (16 h)
- Lung (airway-perfused)
 - Parenchymal blood accumulation (resembles congestion) (40 min)
 - Pyknosis of a majority of alveolar cells (40 min)
 - Separation of bronchiolar epithelium from the lamina propria (1 h)
 - Presence of proteinaceous fluid in alveolar spaces (2 h)
- Lymph node, mandibular
 - Decreased density or rarefaction in cortical lymphocytes (20 min)
 - Cleavage lines resembling "dull-knife distortion" (1 h)
 - Pyknosis of cortical lymphocytes (4 h)
- Lymph node, mesenteric
 - Decreased density or rarefaction in cortical lymphocytes (20 min)
 - Cleavage lines resembling "dull-knife distortion" (1 h)
 - Pyknosis of cortical lymphocytes (2 h)
- Mammary gland (female)
 - Formation of clear spaces at base of acinar epithelial cells (1 h)
 - Pyknosis of acinar epithelium (2 h)
 - Sloughing of epithelial cells into ducts (4 h)
- Mammary gland (male)
 - Increased foamy, vacuolated appearance of acinar cell cytoplasm (4 h)
 - Pyknosis of acinar epithelium (4 h)
- Ovary
 - Formation of clear spaces around follicles (40 min)
 - Individualization of luteal cells (4 h)
- Pancreas, exocrine
 - Vacuolar spaces in acinar epithelium (40 min)
 - Loss of central chromatin (hollow nuclei) in pancreatic duct epithelium (1 h)
 - Formation of clear interstitial spaces around acini (4 h)
 - Disappearance of eosinophilic, proteinaceous luminal duct contents (4 h)
- Parathyroid gland
 - Formation of clear spaces at base of chief cells (1 h)
- Peripheral nerve, sciatic nerve
 - Slight nuclear contraction of Schwann cells (2 h)
- Pituitary gland, pars distalis
 - Increased pyknotic pituicytes (2 h)
 - Pallor or disappearance of pituicyte cytoplasm (4 h)
- Pituitary gland, pars intermedia
 - Increased pyknotic pituicytes (4 h)
 - Pallor or disappearance of pituicyte cytoplasm (4 h)
- Preputial gland
 - No remarkable postmortem changes at 16 h
- Prostate, dorsal
 - Pale or clear luminal contents (<20 min)
- Prostate, ventral
 - Pale or clear luminal contents (16 h)
- Salivary gland, parotid
 - Vacuole and clear space formation in the basal portion of the ductular epithelium (40 min)
 - Pyknosis of ductular epithelium (1 h)
 - Pyknosis and karyoklasis (clumping of chromatin) of acinar epithelium, which resembles very acute necrosis (12 h)
- Salivary gland, sublingual
 - Pyknosis and disruption of ductular epithelium (8 h)
- Salivary gland, submaxillary
 - Loss of tubuloalveolar (serous) cell nuclei (2 h)
 - Tubuloalveolar (serous) cell dispersion that resembles very acute necrosis (2 h)
 - Disintegration and disruption of interlobular duct epithelium that resembles very acute necrosis (4 h)
 - Mucus-producing acini were unremarkable at 16 h
- Seminal vesicles
 - Pyknosis of epithelium (1 h)
 - Detachment and change to cuboidal appearance of epithelial cells with disruption of epithelial surface (2 h)
 - Appearance of fractures and lucent or paler areas in lumen contents (8 h)
- Skeletal muscle
 - Fiber disorganization and "fracturing" into small haphazard cleavage spaces (20 min)

- Formation of interfiber clear spaces (1 h)
- Condensed and hyperchromatic endomysial nuclei (1 h)

Skin

- Chromatin clumping and karyorrhexis of sebaceous cells (40 min)
- Pyknosis of dermal fibrocytes (2 h)
- Epidermis and dermis were unremarkable at 16 h

Spleen

- Pyknosis of megakaryocytes (<20 min)
- Decreased density or rarefaction of lymphocytes in white pulp (40 min)
- Cleavage lines resembling “dull-knife distortion” (40 min)
- Pyknosis of most white pulp lymphocytes (8 h)
- Generalized pyknosis of red pulp (12 h)

Stomach, forestomach

- Pyknosis of basal epithelial cells (4 h)
- Pyknosis of fibrocytes in superficial lamina propria (4 h)
- Formation of clear spaces around fibrocytes in superficial lamina propria (4 h)
- Formation of interfiber clear spaces and pyknosis of myofiber nuclei (8 h)

Stomach, glandular

- Loss of superficial epithelium from mucosal ridges (<20 min)
- Separation of glandular epithelium from basement membrane (40 min)
- Formation of interfiber clear spaces and pyknosis of myofiber nuclei (1 h)
- Separation of crypt epithelium from basement membrane (2 h)
- Lateral separations in glandular epithelium (4 h)
- Pyknosis of crypt epithelium (4 h)

Testes

- Individualization of interstitial cells (1 h)
- Pyknosis of interstitial cells (2 h)
- Formation of clear spaces around seminiferous tubules (8 h)

Thymus

- Large medullary extravasations of erythrocytes, which resemble antemortem thymic hemorrhages and apparently result from the loss of blood from leaky thymic vessels (<20 min)
- Cleavage lines resembling “dull-knife distortion” (4 h)
- Nuclear contraction of cortical lymphocytes (4 h)
- Decreased density or rarefaction in cortical lymphocytes (16 h)

Thyroid gland

- Pale, foamy, and/or bubbly colloid (<20 min)
- Pyknosis of parafollicular “C” cells (40 min)
- Epithelial cells free in colloid (1 h)
- Pyknosis of follicular epithelium (2 h)
- Disruption of follicular epithelium (4 h)
- Pyknosis of epithelium in largest follicles occurred later than in small- to medium-sized follicles (8 h)

Tongue

- Formation of interfiber clear spaces in skeletal muscle (40 min)
- Perinuclear clear spaces (halo) in polyhedral cell layer of the mucosa (4 h)
- Contraction and darkening of muscle cell nuclei (4 h)
- Absence of mitotic figures in basal epithelial cell layer (12 h)

Trachea

- Disruption or sloughing of epithelium into glandular lumen (4 h)
- Pyknosis of superficial epithelium (8 h)
- Loss or sloughing of superficial epithelium (8 h)
- Loss of cilia (12 h)
- Pyknosis of glandular epithelium (12 h)
- Formation of intracellular soap, basophilic deposits in fat cells, deep in the mucosa (16 h)

Urinary bladder

- Formation of vacuoles in transitional epithelium (<20 min)
- Formation of interfiber clear spaces and pyknosis of myofiber nuclei (1 h)
- Disruption and sloughing of the surface transitional epithelium (4 h)

Uterus

- Separation of glandular epithelium from basement membrane (20 min)
- Formation of interfiber clear spaces and pyknosis of myofiber nuclei (4 h)
- Sloughing of superficial endometrial epithelium (8 h)
- Cervix had no remarkable postmortem changes at 16 h

Vagina

- No remarkable postmortem changes at 16 h

HISTOPATHOLOGIC FINDINGS IN CONTROL LABORATORY MICE^{10–32}

Adrenal gland, cortex

- Amyloidosis (amyloid deposition)—associated with systemic amyloidosis (*see* Kidney)
- Atrophy, diffuse—low incidence (CD-1: males 5%, females <1%)
- Accessory cortical nodules—common in adrenal capsule or adjacent connective tissues (CD-1 mice, 2%–5%)
- Extramedullary hematopoiesis—usually occurs in association with leukemoid reactions and myeloid hyperplasia (*see* Bone marrow)
- Pigmentation, ceroid (lipofuscin)—accumulates in residual X-zone cells
- Hyperplasia, eosinophilic cell, focal (CD-1: 2000 males 10.8%, 2000 females 8.8%)
- Hyperplasia, subcapsular (type A) spindle cells: common in mice of all ages; incidence up to 80% in aged CD-1 females (CD-1: 2000 males 16.6%, 2000 females 33.6%)

Hyperplasia, subcapsular vacuolated (type B) cells: proliferation of large polyhedral vacuolated cells; usually associated with spindle cell hyperplasia

X-zone (inner cortex)—normal basophilic cells in young mice of both sexes; involution complete by about 10 days of age in males, may be seen up to 30 weeks of age in females; characterized in females by marked vacuolation, especially in nonbred females

Tumors

Cortical adenoma, all types—low incidence

(CD-1: 891 males 0.9%, 890 females 0.5%)

(CD-1: 2000 males 1.3%, 2000 females <1%)

(B6C3F₁: 2240 males 2.4%, 2306 females 0.3%)

Type A cell adenoma

Type B cell adenoma

Cortical carcinoma (adenocarcinoma), all types: very low incidence

(B6C3F₁: 3 reported in 2240 males 0.1%, 1 reported in 2306 females <0.1%)

(CD-1: none in 891 males, 3 in 890 females 0.3%)

(CD-1 mice: 2000 males none, 2000 females <1%)

Adrenal gland, medulla

Hyperplasia—low incidence (CD-1 mice, 1%–8%; males > females)

Hypertrophy

Tumors

Ganglioneuroma—very rare; none reported in more than 10,000 CD-1 and B63F₁ mice

Pheochromocytoma (benign)—low incidence

(CD-1: 5 in 891 males 0.6%, 3 in 890 females 0.3%)

(CD-1 mice: 2000 males <1%, 2000 females <1%)

(B6C3F₁: 2240 males 1.2%, 2306 females 0.7%)

Pheochromocytoma, malignant—very low incidence

(CD-1: none in 891 males, 1 in 890 females 0.1%)

(B6C3F₁: 2 reported in 2240 males 0.1%, none reported in 2306 females 0.0%)

Blood vessels

Amyloidosis—frequent vascular finding especially in lungs and liver (*see* Kidney).

Angiectasis—common finding especially in uterus, ovary, liver, spleen, and lymph nodes.

Arteritis (polyarteritis nodosa, periarteritis, perivascularitis)—frequent finding, most often in the kidney, heart, mesentery, uterus, testis, and urinary bladder (RF: 311 females 18.0%).

Hyalinization, arterial—amyloid-negative eosinophilic deposits within the media of arterial walls (RF: 311 females 14.1%).

Mineralization—foci of calcification in the wall of the aorta are common in some strains such as DBA (*see* Heart).

Tumors

Hemangioma and hemangiosarcoma—common especially in the subcutaneous tissue, liver, spleen, and uterus.

Hemangioma (angioma, hemangioendothelioma), all sites (B6C3F₁: 2343 males 1.5%, 2486 females 1.6%).

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma), all sites (B6C3F₁: 2343 males 2.7%, 2486 females 1.9%).

Bone

Exostosis (hyperostosis) (*see* Hyperplasia)

Fracture callus—common in ribs and extremities, especially feet and tail from restraining or fighting

Hyperplasia (exostosis, hyperostosis)—sporadically associated with chronic infections; observed also as a focal thickening of ventral thoracic vertebrae from gavage

Inflammation (osteomyelitis)—common in bones of feet and tail associated with wounds from fighting

Necrosis, sternal—reported in sternbrae of nearly all of 427 female RF mice older than 15 months of age

Osteodystrophy (fibro-osseous lesions) common in B6C3F₁ female mice; incidence up to 40% in females and <1% in males (B6C3F₁: 2543 males none, 2522 females 1.0%)

Osteitis fibrosis—uncommon, associated with hyperparathyroidism and severe renal disease

Tumors

Ossifying fibroma—occasional, reported in jaw of CD-1 mice

Osteoma, all sites—uncommon in CD-1 mice (CD-1: 1 in 891 males 0.2%, 2 in 890 females 0.2%)

Osteosarcoma, all sites (CD-1: 2 in 891 males 0.2%, 2 in 890 females 0.2%) (B6C3F₁: 2343 males 0.1%, 2486 females 0.6%)

Bone marrow

Atrophy (hypoplasia, aplasia)—unusual finding

Fibrosis—occasional small foci in the marrow space

Hyperplasia, erythroid—associated with anemia

Hyperplasia, megakaryocytic—usually accompanies myeloid hyperplasia

Hyperplasia, myeloid (leukemoid reaction)—associated with neutrophilia, abscesses, acute pyelonephritis, pneumonia, extensive necrosis, neoplasms, anemia, and systemic infections; accompanied by extramedullary hematopoiesis

Tumors

Leukemia, myelogenous (myeloid, granulocytic)

(CD-1: 891 males 1.8%, 890 females 1.9%)

(B6C3F₁: 2343 males 0.7%, 2486 females 2.1%)

Brain and meninges (central nervous system [CNS])

Cyst, epithelial—squamous cell cysts are uncommon; most often observed in the spinal cord.

Chromatolysis—occasional finding characterized by dispersion and dissolution of the Nissl substance.

Dark cells—common finding; foci of neurons that appear shrunken and basophilic without changes in the surrounding parenchyma or glial cells are regarded as artifacts.

Gitter cells—microglial macrophages usually associated with myelin breakdown.

Infarction—uncommon focal finding as a result of vascular blockage, usually observed as a healing area of chronic inflammation.

Inflammation—frequent finding as small foci of chronic inflammatory cells, lymphocytes, and plasma cells in the brain and meninges.

Malacia—necrosis of neural tissues with loss of architecture, breakdown of myelin or neuropil, and accumulation of foamy macrophages (gitter cells).

Mineralization, cerebral—common small basophilic deposits; usually in thalamus; in up to 5% of CD-1 mice.

Necrosis—ischemic neuronal injury, occasional finding of shrunken, hyaline, densely eosinophilic cell bodies and small, basophilic nuclei that results from a variety of causes including ischemia, anoxia, seizures, epilepsy, and some metabolic disturbances.

Pigmentation, melanin (melanosis)—common in meninges of olfactory bulb and optic nerves of pigmented strains.

Vacuolation—vacuoles are a frequent finding associated with a variety of causes including artifacts, intramyelinic edema, spongy degeneration, spongiform encephalopathies, uremia, retrovirus (murine leukemia virus) infection, and probably aging.

Tumors

CNS tumors are rare in most mice; usual incidence 0.05%–0.1% (B6C3F₁: 2303 males none, 2378 females none).

Astrocytoma—rare but most reported in VM and BRVR strains, up to 1.5% (B6C3F₁: 2849 males none, 2826 females none).

Lipoma—uncommon lesion consisting of lipocyte foci in the choroid plexus reported in BALB/c, and other strains may be a hamartoma (malformation) not neoplastic (B6C3F₁: 1 in 2849 males 0.04%, 2826 females none).

Meningioma—uncommon but reported in CD-1, in B6C3F₁, and most often in C3H strains. (CD-1: 1 in 891 males 0.1%, none in 890 females) (B6C3F₁: 2849 males none, 1 in 2826 females 0.04%)

Oligodendroglioma—rare finding, most reported in BALB/c (B6C3F₁: 1, malignant, in 2849 males 0.04%; 1, benign, in 2826 females 0.04%).

Clitoral gland

Small in mice; often examined only if grossly abnormal.

Atrophy—minor degrees are common in aged mice.

Cysts—minor degrees are common in aged mice.

Dilatation—commonly, cysts accompany atrophy.

Enlargement—commonly a result of cysts or inflammation.

Inflammation, suppurative (abscess)—common finding.

Tumors—none reported (B6C3F₁: 2486 females none).

Coagulating gland

Inflammation, suppurative—usually associated with prostatic, urinary bladder, or kidney inflammation

Tumors

Adenoma—uncommon finding

(CD-1: 3 in 891 males 0.3%)

(B6C3F₁: none in 2343 males)

Adenocarcinoma—rare; none in CD-1 mice (B6C3F₁: none in 2343 males)

Ductus (vas) deferens (*see* Epididymis)

Ear

External ear (pinna or auricle and auditory canal)

Inflammation, auricular cartilage (proliferative chondritis)

Inflammation, chronic—frequent finding as a result of wounds and fighting (*see* Skin for additional lesions)

Tumors—same as Skin

Middle ear

Inflammation (otitis media)—occasional finding, often associated with sinusitis and upper respiratory infections; tympanic cavity usually contains purulent exudate.

Inner ear

Inflammation (otitis interna)—often extending from the middle ear; can result in encephalitis and loss of balance or circling behavior; may be associated with active necrotizing arteritis in adjacent tissues

Zymbal's gland (auditory sebaceous glands)

Tumors—none reported in CD-1 mice (B6C3F₁: 1, adenoma, in 2855 males 0.04%; 1, carcinoma, in 2838 females 0.04%)

Epididymis

Changes commonly observed are secondary to testicular atrophy and include lack or diminished spermatozoal contents, intraepithelial cysts formation, and tubular debris.

Inflammation, granulomatous (spermatic granuloma)—common.

Tumors—very rare

Hemangioma (angioma, hemangioendothelioma)—CD-1: 1 in 891 males 0.1%.

Leiomyoma—one has been reported in a B6C3F₁ mouse.

Sarcoma—3 (0.1%) reported in 2823 B6C3F₁ mice.

Esophagus

Inflammation—common in association with gavage injuries during dosing; severity and frequency range from small foci of macrophages to large areas of necrosis and abscess formation; perforations can lead to extensive inflammation of subcutis or pleura.

Tumors—unusual; squamous cell tumors have been reported in some strains (B6C3F₁: 1 papilloma in 3789 females 0.03%, 1 carcinoma in 2855 males 0.04%, and 1 carcinoma in 2838 females 0.04%).

Eye

Atrophy, retina—frequent finding from a variety of causes including genetics, light exposure, and retro-orbital

blood sampling; low incidence in CD-1; high incidence in C3H (up to 100%), CBA, Swiss.

Cataract, lens—very common finding (over 25%) in CD-1 mice based on ophthalmologic examinations.

Degeneration, retina—associated with atrophy.

Dystrophy, corneal (*see* Mineralization).

Inflammation, globe (panophthalmitis)—occasional finding; usual intraocular findings consist of small lymphocyte and plasma cell infiltrates in ciliary body and sclera; more severe after retro-orbital blood sampling; most severe after fight wounds; may result in phthisis bulbi (severe atrophy).

Inflammation, conjunctiva (conjunctivitis)—suppurative inflammation occurs sporadically, especially abscesses of meibomian glands.

Inflammation, cornea (keratitis)—sporadic occurrence of acute or chronic inflammation results in opacities.

Opacity, cornea (*see* Inflammation and Mineralization).

Mineralization, cornea (corneal dystrophy)—subepithelial mineralization of the cornea is a common finding in CD-1 mice with an incidence up to 10%; also increased in some strains such as DBA, BALB/c, and C3H (*see* Heart).

Iris, mineralization—sporadic finding in CD-1 mice.

Phthisis bulbi—shrinkage or severe atrophy of the globe; usually the result of chronic inflammation or trauma.

Tumors—intraocular tumors are very rare in mice.

Forestomach

Erosion (*see* Ulcer)

Diverticulum—rare finding

Ulcer—erosions and ulcers (focal necrosis) with accompanying inflammation of the squamous mucosa are very common lesions; vary in severity; severe ulceration is accompanied by acute and chronic inflammation, penetration of the inflammatory changes into the stomach wall, and in some animals, peritonitis.

Hyperkeratosis—associated with hyperplasia; occurs as a diffuse change in anorectic mice.

Hyperplasia, squamous (acanthosis, hyperkeratosis, parakeratosis)—most proliferative or hyperplastic changes of the squamous mucosa are reactive, focal, and associated with gastric inflammation; occasionally the entire forestomach is affected; focal hyperplasia commonly involves the junction with the glandular stomach.

Inflammation (gastritis)—common finding often associated with erosion or ulceration; vary from focal acute or chronic inflammation to perforation of the stomach wall; foci of lymphocytes and plasma cells in the submucosa are common.

Necrosis—superficial necrosis (erosion) and deep necrosis (ulceration) are common findings (*see* Ulcer).

Tumors—squamous cell tumors are common:

Squamous papillomas—low incidence; up to 1% in CD-1 mice

(B6C3F₁: 2252 males 0.3%, 2486 females 0.5%)

(B6C3F₁: 3807 males 1.6%, 3789 females 3.6%)

Squamous carcinomas—very low incidence

(CD-1: 3 in 891 males 0.3%, 1 in 890 females 0.1%)

(B6C3F₁: none in 2252 males, 2 in 2486 females 0.1%)

(B6C3F₁: 3807 males 0.2%, 3789 females 0.2%)

Leiomyoma—very low incidence (B6C3F₁: none in 3807 males, 2 in 3789 females <0.1%)

Mast cell tumor (B6C3F₁: 2, benign, <0.1% and 1, malignant, <0.1% in 3807 males; 1, benign, in 3789 females <0.1%)

Gallbladder

Degeneration, hyaline—eosinophilic droplets are commonly present in epithelial cells of aged B6C3F₁ mice.

Inflammation (cholecystitis)—foci of inflammatory cells occasionally occur within the walls of bile ducts and the gallbladder.

Tumors—unusual

(B6C3F₁: 2 adenomas 0.08% and 1 sarcoma 0.04% in 2484 males; 1 papilloma in 2590 females 0.04%);

(CD-1: papillomas, 1 in 891 males 0.1%; 1 in 890 females 0.1%)

Harderian gland

Atrophy—common focal or diffuse finding associated with cystic dilatation of glandular lumens.

Cysts—occasional finding.

Ectasia, glandular (cystic dilatation)—focal or diffuse dilatation of glandular lumens with epithelial atrophy.

Hyperplasia, focal—occasional finding of small, non-compressive lesions with both cellular hyperplasia and hypertrophy.

Inflammation (adenitis)—focal inflammatory cells, especially small aggregates of lymphoid cells, are common; 20% incidence was observed in 274 C57BL/6 female mice; incidence increases with age.

Tumors

Adenoma—low incidence

(<2% of CD-1 mice, up to 8% of B6C3F₁, <3% of C3H)

(CD-1: 891 males 10.2%, 890 females 6.95%)

(B6C3F₁: 2343 males 2.1%, 2486 females 1.3%)

Adenocarcinoma—very low incidence (<1% of CD-1 mice)

(CD-1: 891 males 0.5%, 890 females 0.2%)

(B6C3F₁: 2 reported in 2343 males 0.1%, 1 reported in 2486 females <0.1%)

Myoepithelioma—infrequent in most strains but more common in BALB mice, especially females

Heart

Amyloidosis—common finding in CD-1 mice; often associated with cardiac atrial thrombosis (*see* Kidney).

Atrial thrombosis—common finding associated with pulmonary chronic venous congestion.

(RF: 427 females 6.8%)

(CD-1: 2000 males 4.5%, 2000 females 1%)

Fibrosis—usually associated with necrosis (CD-1: 2000 males <1%, 2000 females <1%).

Inflammation (myocarditis)—minor aggregates of inflammatory cells and fibrosis are common; more severe changes are usually associated with necrosis and infectious diseases (CD-1: 2000 males 3.4%, 2000 females 2.5%).

Mineralization (dystrophic cardiac or myocardial calcification, cardiac calcinosis)—the heart as well as the tongue, cornea, and aorta are frequent sites of soft-tissue calcification in some strains: DBA, C3H, BALB/c, CBA, and C3N with incidence of 100% in DBA/2; uncommon in CD-1 and B6C3F₁ mice; incidence and severity are modified by age, sex, parity, and diet.

Myocardial degeneration—occasional finding occurring as vacuolation of myocytes.

Myocardial necrosis (focal necrosis; acute, necrotizing inflammation; acute focal inflammation; focal sub-acute or chronic inflammation; focal fibrosis)—a frequent localized lesion accompanied by inflammatory cells and fibrosis; incidence at 18 months of age, 30%–40% in CD-1.

Pigmentation, melanin (melanosis)—common in heart valves of pigmented strains, such as C57BL.

Valvular inflammation—uncommon.

Tumors

Hemangioma (angioma, hemangioendothelioma)—very rare (B6C3F₁: none in 3807 males, none in 3789 females).

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—very rare (B6C3F₁: 3798 males <0.1%, 3779 females <0.1%).

Mesothelioma (CD-1: 1 in 891 males 0.1%, none in 890 females).

Rhabdomyosarcoma—very rare.

Secondary metastatic or multiple systemic tumors are common.

Intestines (unspecified, NOS)

Amyloidosis—frequent finding in CD-1 mice, especially in jejunum (*see* Kidney).

Arteritis—occasional finding in mesenteric blood vessels of CD-1 mice; fibrinoid necrosis usually present.

Ectopic pancreatic tissue—sporadic finding in duodenum.

Dilatation—frequent finding in animals found dead, probably from bacterial gas production; dilatation of the colon, occasional finding of unknown cause.

Hyperplasia, lymphoid—occasional finding from immunological stimulation such as systemic illness (CD-1: 2000 males 1%, 2000 females 1%).

Hyperplasia, mucosal—normal physiological thickening of the intestines occurs in pregnant and lactating mice.

Metazoan parasites.

Nematodiasis—pinworms are common findings in the colon and occasionally in the ileum with no inflammation.

Tumors

Adenoma—very low incidence, <0.3% in CD-1 mice; most often in duodenum

Adenoma, small intestine

(CD-1: 2 in 891 males 0.2%, 1 in 890 females 0.1%)

(B6C3F₁: 3807 males 0.8%, 3789 females 0.4%)

Adenomatous polyp, small intestine (B6C3F₁: 3807 males 0.2%, 3789 females 0.1%)

Adenoma, anus (CD-1: 1 in 891 males 0.1%, none in 890 females)

Adenocarcinoma—very low incidence, <0.1% in CD-1 mice

Adenocarcinoma, small intestine (CD-1: none in 891 males, 2 in 890 females 0.2%)

(B6C3F₁: 2148 males 0.7%, 2 in 2234 females 0.1%)

(B6C3F₁: 3807 males 1.0%, 3789 females 0.3%)

Adenocarcinoma, large intestine (B6C3F₁: 1 in 3807 males <0.1%, none in 3789 females)

Leiomyoma—occasional finding

Leiomyoma, large intestine (B6C3F₁: none in 3807 males, 2 in 3789 females <0.1%)

Leiomyosarcoma—occasional finding

Leiomyosarcoma, large intestine (B6C3F₁: none in 3807 males, 2 in 3789 females <0.1%)

Sarcoma, unspecified—occasional finding

Sarcoma, large intestine (CD-1: 1 in 891 males 0.1%, none in 890 females)

Squamous cell carcinoma—occasional finding

Squamous cell carcinoma, large intestine (CD-1: none in 891 males, 1 in 890 females 0.1%)

Squamous cell carcinoma, anus (CD-1: 1 in 891 males 0.1%, none in 890 females)

Squamous cell carcinoma, rectum (B6C3F₁: 1 in 3807 males <0.1%, none in 3789 females)

Islets, pancreatic

Atrophy, beta cell—diabetes mellitus is rare in CD-1 mice; genetically determined occurrence in some strains, for example, db/db.

Hyperplasia—uncommon in CD-1 mice, common in C3H; large islets are common in aged B6C3F₁ mice.

Tumors

Islet cell adenoma—low incidence, 0.5% in CD-1 (CD-1: 891 males 0.3%, 890 females 0.6%) (B6C3F₁: 2237 males 0.4%, 2200 females 0.4%)

Islet cell carcinoma—uncommon in CD-1 mice (B6C3F₁: none, 2237 males, 2 in 2200 females 0.1%)

Joints

Arthrosis—common in CD-1 mice

Inflammation (arthritis)—occasional lesions, usually adjacent to wounds or septic foci

Osteoarthritis—degenerative lesions with inflammation; low incidence (0–5%, CD-1 mice)

Subluxation—occasional lesion of intervertebral joints resulting in distortion of the tail

Prolapsed intervertebral disk—rare, reported in CD-1 mice

Kidney

Amyloidosis—common site, usually associated with systemic amyloidosis; additional sites include intestines, myocardium, nasal submucosa, parotid salivary gland, thyroid gland, adrenal cortex, spleen, lungs, liver,

tongue, testes, ovary, uterus, aorta, skin, pancreas, and lymph node (primarily mesenteric); considered cause of illness or death in 6.5% of 766 male and 8.1% of 766 female CD-1 mice over 12 months of age; various incidences (CD-1 mice, 10%–40%; B6C3F₁, 2543 males 1.9%, 2522 females 0.2%).

Cysts (polycystic disease)—common finding associated with dilatation of tubules; cortical cysts are common in CD-1; congenital cysts of varying size have high frequencies in several strains such as BALB.

End stage—marked or severe glomerular, tubular, and interstitial changes with inflammation.

Extramedullary hematopoiesis—usually occurs in the perirenal fat associated with leukemoid reactions and myeloid hyperplasia (*see* Bone marrow).

Glomerulonephritis (glomerulonephropathy, glomerulosclerosis)—common finding in certain strains of older mice such as AKR, BALB/c, and CBA; low incidence as significant lesion in most strains (RF: 427 females 84%, 311 females 95.1%)

(CD-1: males 4%, females 12%)

(CD-1: 2000 males 24.9%, 2000 females 24.9%)

(B6C3F₁: 2543 males 1.2%, 2522 females 2.4%)

Hydronephrosis—common finding; can occur in high prevalence among certain strains or be secondary to urinary obstruction or pyelonephritis; various incidences (CD-1 mice, up to 10%; B6C3F₁, 2543 males 0.6%, 2522 females 0.2%)

Hyperplasia, tubular epithelium, focal—occasional finding.

Infarction—common finding; low incidence (CD-1 mice: 2%–4%).

Inflammation, nonsuppurative—common finding of lymphocyte aggregates that tend to accumulate around renal interlobular arteries; 3% incidence was observed in 274 C57BL/6 female mice.

Interstitial (tubulointerstitial) nephritis—tubular degeneration and interstitial fibrosis with lymphocytic infiltrates and sometimes mineral deposits (CD-1: males 5.5%, females 3.8%).

Mineralization (nephrocalcinosis, calcification)—small foci of mineral deposits are common (CD-1: males 5.0%, females 2.4%).

Necrosis, tubular—kidneys of mature male mice of certain genotypes such as DBA and C3H are exquisitely sensitive to chloroform fumes that result in tubular necrosis and mineralization.

Obstructive uropathy (urologic syndrome, obstructive genitourinary disease) (*see* Urethra).

Papillary necrosis—usually occurs in kidneys with severe damage associated with amyloidosis.

Pyelonephritis—occasional finding in CD-1 mice, usually associated with inflammation of the lower urinary tract, including pyelonephritis in males that accompanies inflammation of the prostate, seminal vesicles, coagulation gland, and urinary bladder; also occurs in some animals with urethral mucous plugs; embolic

suppurative nephritis is infrequent (B6C3F₁: 2543 males 1.6%, 2522 females 1.5%).

Tumors

Adenoma—very low incidence (<1%; CD-1 mice) (CD-1: 3 in 891 males 0.3%, 1 in 890 females 0.1%) (B6C3F₁: 3791 males 0.3%, 3767 females 0.1%)

Adenocarcinoma—very low incidence (<0.1%; CD-1 mice)

(CD-1: 1 in 891 males 0.1%, 1 in 890 females 0.1%)

Carcinoma—very rare

(CD-1: 2 in 891 males 0.2%, none in 890 females)

(B6C3F₁: 1 in 3791 males <0.1%, 1 in 3767 females <0.1%)

Lipoma—very rare (CD-1: 1 in 891 males 0.1%, none in 890 females)

Nephroblastoma—very rare; none reported in CD-1 mice

Transitional cell carcinoma—very rare in mice

(CD-1: none in 891 males, none in 890 females)

(B6C3F₁: 1 in 244 males 0.4%, none in 246 females)

Lacrimal gland—ectopic Harderian gland tissue, occasional finding (*see* Harderian gland)

Larynx (*see* Trachea)

Liver

Amyloidosis—common site in systemic amyloidosis; occurs in 10%–20% of CD-1 mice in blood vessels and along sinusoids (*see* Kidney).

Angiectasis (telangiectasis)—common finding, especially in females, up to 10% of CD-1 mice; widely dilated vascular spaces lined by normal-appearing endothelium that results in blood-filled cystic spaces and distortion of the sinusoids (also *see* Sinusoidal dilatation); incidence reported as telangiectasis (CD-1: 2000 males <1%, 2000 females <1%).

Congestion—common finding dependent on the mode of death and length of time before tissue fixation; frequently centrilobular and more pronounced after cardiac failure.

Cytomegaly—common finding in hepatocytes of aged mice.

Dilatation, sinusoidal, focal—common in aged mice; consists of widely dilated sinusoids, sometimes cystic, which retains the shape and position of the sinusoids.

Enlargement (hepatocytic hypertrophy).

Extramedullary hematopoiesis—frequent finding as foci along sinusoids or around central veins and portal vessels; usually associated with leukemoid reactions and myeloid hyperplasia (*see* Bone marrow).

Fatty change (fatty metamorphosis, lipidosis)—fat vacuoles in hepatocytes occur in 5% of CD-1 mice; usually midzonal in untreated mice and increases with age and obesity; may accompany hepatocytic cellular alteration; focal cytoplasmic vacuolative changes of hepatocytes adjacent to the cleft of the median lobe have been termed tension lipidosis; microvesicular fatty

- change has been associated with Reye-like syndrome in BALB/c ByJ mice.
- Hepatocytic cellular alteration, focal—occasional finding of foci from one to several lobules in diameter consisting of hepatocytes; identified as clear cell, eosinophilic, basophilic, and mixed cell foci (CD-1: 2000 males <1%, 2000 females <1%).
- Hepatocytic, degeneration, vacuolation (may be included in some reports as fatty change; see fatty change) (CD-1: 2000 males 2.5%, 2000 females 2%).
- Hyperplasia, hepatocellular (nodular)—occasional finding of diffuse or focal areas larger than a lobule consisting of normal or hypertrophic hepatocytes (CD-1: 2000 males 1.3%, 2000 females <1%).
- Hypertrophy, hepatocytic—common finding in aged mice associated with cytomegaly, karyomegaly, and intranuclear inclusions.
- Inclusion bodies, intranuclear—common finding of eosinophilic inclusions in the nuclei of hepatocytes resulting from invaginations of the cytoplasm; increased with age.
- Inclusion bodies, cytoplasmic—common finding of eosinophilic inclusions in the cytoplasm of hepatocytes; increased with age.
- Inflammation (hepatitis)—very common finding (CD-1: 2000 males 19.3%, 2000 females 9.3%); up to 50% of CD-1 mice have small foci of both acute and chronic inflammatory cells scattered in the liver; small aggregates of lymphoid cells are common; 20% incidence was observed in 274 C57BL/6 female mice; larger foci are associated with coagulative necrosis that may be related to *Bacillus piliformis* (Tyzzer's disease) or mouse hepatitis virus occurs in up to 5% of CD-1 mice of some laboratories; acute, chronic, and necrotic hepatitis can result from *Helicobacter hepaticus* infections.
- Karyomegaly—common finding in hepatocytes of aged mice; usually associated with anisokaryosis, polykaryia, and cytoplasmic invagination into the nucleus (inclusions).
- Necrosis—in addition to the necrosis associated with inflammation, necrotic lesions characterized by fibrous repair, round cell infiltration, oval cell proliferation, and usually hemosiderin deposition occur occasionally in untreated mice; individual cell necrosis of single or small aggregates of hepatocytes is a sporadic finding.
- Necrosis, hepatocellular (hepatocytic)—focal necrosis of unknown cause occasionally occurs in weanling mice; incidence <2% reported in 445 B6C3F₁ males and females (CD-1: 2000 males 3.5%, 2000 females 9.7%).
- Pigmentation—lipofuscin (ceroid) and hemosiderin are occasionally present in Kupffer cells.
- Telangiectasis (*see* Angiectasis).
- Tumors—frequency varies greatly within strains and between strains; hepatocellular tumors can increase after infection by *H. hepaticus*
- Hemangioma (angioma, hemangioendothelioma)—low incidence, 2%–4% of CD-1 mice (CD-1: 891 males 1.5%, 890 females 0.5%).
 - Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—low incidence, <0.4% in CD-1 mice (CD-1: 4 in 891 males 0.5%, 1 in 890 females 0.1%).
 - Hepatoblastoma—rare finding; often associated with hepatocellular carcinomas; may be undifferentiated variant of hepatocellular carcinoma.
 - Hepatocellular adenoma—very common (CD-1: males 31%, females 3.5%) (CD-1: 2000 males 5.2%, 2000 females 1.7%) (B6C3F₁: males 10%, females 4%) (B6C3F₁: 2334 males 10.3%, 2469 females 4%)
 - Hepatocellular carcinoma (adenocarcinoma) (CD-1: males 13%; females 0.5%) (CD-1: 2000 males 4.4%, 2000 females <1%) (B6C3F₁: males 21%, females 5%) (B6C3F₁: 2334 males 21.3%, 2469 females 4.1%)
 - Leukemia, myelogenous (myeloid, granulocytic)—often involves mainly liver and spleen in RF strain.
 - Malignant lymphoma—lymphomas are commonly perivascular, either around portal triads or central veins.
 - Metastatic tumors—common site for secondary and metastatic malignant tumors, especially lymphoreticular neoplasms.
- Bile ducts**
- Cholangiofibrosis (adenofibrosis)—uncommon finding; characterized by excessive proliferation of fibrous tissue and bile ducts
 - Inflammation (cholangitis)—occasional finding of acute inflammatory cells increases with age; frequently accompany bile duct hyperplasia and peritubular fibrosis
 - Hyperplasia—common in old mice accompanied by peritubular fibrosis and inflammatory cells
 - Tumors—very rare in mice
 - Cholangioma—one reported in CD-1 mice
 - Cholangiosarcoma—one reported in CD-1 mice
- Lung**
- Abscess—occasional finding, usually in aged animals; results from local or systemic infections.
 - Bronchiectasis—dilatation of bronchi or bronchioles usually results from infections with chronic inflammation; may be accompanied by squamous metaplasia.
 - Bronchopneumonia—occasional finding in old mice; frequent in younger animals on gavage experiments (RF: 427 females 9.4%).
 - Congestion—frequent finding associated with agonal changes related to the mode of death; marked in animals found dead; accompanied by edema in cardiac failure.
 - Chronic venous congestion—occasional finding associated with thrombosis of the left atrium; accompanied by alveolar septa thickening and fibrosis (fibrosing alveolitis or interstitial pneumonia).
 - Crystal pneumonitis—sporadic focal accumulations of eosinophilic crystals in terminal airways and alveolar sacs in some strains, especially C57BL.

Degeneration, hyaline—eosinophilic droplets are commonly present in bronchiolar epithelial cells of aged B6C3F₁ mice.

Edema—sporadic accompanying cardiac failure.

Fibrosis—occasionally found as focal lesions in association with chronic inflammation; diffuse segmental fibrosis (fibrosing alveolitis) secondary to chronic venous congestion.

Hemorrhage—frequent finding associated with agonal changes related to the mode of death or method of sacrifice.

Hyperplasia, alveolar epithelium, focal (adenomatous hyperplasia, pulmonary adenomatosis, alveolar cell hyperplasia, epithelialization)—frequent finding, usually multicentric, increases with increased age.

Hyperplasia, alveolar macrophage (foam cells, histiocytosis, lipidosis)—occasional finding of collections of foamy alveolar macrophages in older mice that may be associated with chronic or granulomatous inflammation and cholesterol clefts.

Hyperplasia, goblet cell—hypersecretion by mucous cells of the bronchial epithelium associated with chronic inflammation.

Hyperplasia, lymphoid—accumulations of lymphoid cells are often located in the visceral pleura within septal clefts; increased lymphoid cells are common in association with inflammation and respiratory infections.

Inflammation, acute and chronic (alveolitis, bronchitis, bronchopneumonia, pneumonia)—very common to have small foci of inflammatory cells, predominantly lymphocytes in occasional alveoli or around bronchioles and small vessels; especially common in females (C57BL/6: 274 females 51%) (C3H: 298 males 4%).

Inflammation, granulomatous (granuloma)—occasional finding resulting from infections, inhaled foreign bodies, and aggregates of alveolar (foamy) macrophages with cholesterol deposits.

Metaplasia, squamous cell—common in bronchi in association with chronic inflammation.

Tumors

Alveolar/bronchiolar adenoma and carcinoma—very common with more in males than females; at 18–24 months, 25%–30% in CD-1 males

Alveolar/bronchiolar adenoma

(CD-1: 891 males 15%, 890 females 14%)

(B6C3F₁: 2328 males 12.1%, 2388 females 5.5%)

Alveolar/bronchiolar carcinoma

(CD-1: 891 males 19%, 890 females 12.1%)

(B6C3F₁: 2328 males 5.1%, 2388 females 2%)

Squamous cell carcinoma—very rare

Hemangiosarcoma (hemangioendothelioma)—very rare

Malignant lymphoma—commonly involves lung around the bronchi, bronchioles, and blood vessels

Metastatic tumors—common site for metastatic malignant tumors especially liver, up to 5% of hepatocellular carcinomas and mammary gland adenocarcinomas

Lymph node (unspecified, NOS): mandibular and mesenteric are most frequently examined:

Amyloidosis—common especially in mesenteric lymph nodes (*see* Kidney) (CD-1: 2000 males 18.1%, 2000 females 18.1%)

Arteritis (CD-1: 2000 males 0.3%, 2000 females 0.3%)

Atrophy—common in aged mice

Congestion—common especially in mesenteric lymph nodes

Extramedullary hematopoiesis—usually occurs in association with leukemoid reactions and myeloid hyperplasia (*see* Bone Marrow); frequent in mice

Hemorrhage—frequent

Hyperplasia, lymphoid (follicular hyperplasia)—common; usually associated with an active inflammatory lesion elsewhere in the body; nonneoplastic proliferation of lymphocytes accompanied by plasma cells and other inflammatory cell types (CD-1: 2000 males 15.3%, 2000 females 15.3%)

Hyperplasia, histiocytic—a form of lymphoid hyperplasia in which histiocytes are predominant cell type

Hyperplasia, plasma cell—common especially in mesenteric and cervical lymph nodes

Inflammation (lymphadenitis) (CD-1: 2000 males 1.2%, 2000 females 1.2%)

Lymphadenomegaly (mesenteric disease)—sporadic in aging mice of various strains, especially C3H; characterized by atrophy of lymphoid tissue and congestion of sinuses

Sinus histiocytosis—common finding in which the sinuses contain active histiocytes and numerous free macrophages

Tumors

Lymphoreticular neoplasms have a high spontaneous incidence in mice.

Hemangiomas and hemangiosarcomas—low incidence (CD-1: 2000 males 0.7%, 2000 females 0.7%).

Hemangioma (angioma, hemangioendothelioma)—B6C3F₁: 2 in 3807 males <0.1%, 2 in 3789 females <0.1%.

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—B6C3F₁: 3807 males 0.2%, 1 in 3789 females <0.1%.

Histiocytic sarcoma (reticulum cell sarcoma, type A)—rare before 12 months; common especially after 18 months of age; frequently the mesenteric lymph node appears to be site of origin; may occur early as single lesions in the mesenteric lymph node, other lymph nodes, Peyer's patches, liver, and spleen; thymus may be involved secondarily; frequently involves the uterus, vagina, and liver and less often the bone marrow, lungs, pancreas, dermis, mesentery, kidney, and epididymis (RF: reticulum cell sarcoma, 427 females 56.2%) (CD-1: 2000 males 2.5%, 2000 females 2.5%).

Leukemia, myelogenous (myeloid, granulocytic)—often secondarily involves lymph nodes

(RF: 427 females 0.9%)

(CD-1: 2000 males 3%, 2000 females 3%)

(B6C3F₁: 2343 males 0.7%, 2486 females 2.1%).

Leukemia, lymphatic (lymphocytic, lymphoblastic)—malignant lymphomas that have disseminated into the peripheral blood.

Malignant lymphoma (lymphosarcoma)—lymphomas have been classified several ways, both on site of primary involvement or organ distribution and cell type; thymic lymphomas reportedly arise in the thymus of some strains of mice such as RF, then primarily involve the mediastinal organs, with subsequent spread to other organs, including the lungs, heart, liver, spleen, ovaries, uterus, and peripheral lymph nodes (also *see* Thymus) (RF: thymic lymphoma, 427 females 4.0%).

Nonthymic lymphoma (lymphosarcoma) reportedly arises as primary neoplasms in one or more lymph nodes, with spread to the spleen and liver; rare or little involvement of the thymus, lung, and heart; and occasional involvement of the ovary, uterus, kidney, and blood in some strains of mice such as RF (RF: nonthymic lymphoma, 427 females 7.0%).

Lymphoma, lymphocytic (small cell lymphoma): occurs most commonly in spleen, lymph nodes, thymus, liver, pancreas, lungs, and bone marrow; if bone marrow involvement is extensive, it becomes leukemic.

Lymphoma, pleomorphic (follicular center cell lymphoma: reticulum cell sarcoma, type B)—rare before 12 months; frequent after 18 months in CD-1 mice; usually arises in Peyer's patches of small intestine, in mesenteric lymph nodes, or in the spleen; also involves other lymph nodes, liver, and bone marrow.

Lymphoma, undifferentiated (lymphoblastic lymphoma, immunoblastic lymphoma, follicular center cell lymphoma; reticulum cell sarcoma, type B).

Lymphoma, mixed type.

Malignant lymphoma (lymphosarcoma), all types

(CD-1: 2000 males 5.7%, 2000 females 5.7%)

(CD-1: 891 males 8.0%, 890 females 22.0%)

(B6C3F₁: 2343 males 12.0%, 2486 females 25.1%).

Mast cell tumor (mastocytoma)—rare; usually involves the liver, spleen, kidneys, and bone marrow

(CD-1: 2 in 891 males 0.2%, none in 890 females).

Mast cell tumor, benign (B6C3F₁: none in 3766 males, 1 in 3751 females <0.1%).

Mast cell tumor, malignant (B6C3F₁: 1 in 3766 males <0.1%, none in 3751 females).

Plasma cell tumor (plasmacytoma, plasma cell lymphoma)—very low incidence in several strains; occurs in lymph nodes, spleen, liver, and bone marrow.

Mammary gland

Cystic change (duct ectasia)—frequent finding often in the absence of hyperplasia in aged mice; incidence as high as 75% in CD-1 mice.

Hyperplasia of duct epithelium and/or alveoli—common but less than duct ectasia in CD-1 mice.

Inflammation (mastitis)—foci of inflammatory cells are an occasional finding. More severe inflammation including abscesses is usually associated with skin wounds or tumors.

Tumors

Incidence varies with strain; CD-1 strain has a low spontaneous incidence of mammary tumors, 5%–7%.

Adenoma—rare in CD-1 and B6C3F₁ mice

(CD-1: 1 in 891 males 0.1%, 890 females 1.0%)

(B6C3F₁: none in 2343 males, 2486 females 0.3%)

(B6C3F₁: 3 in 2522 females 0.1%).

Adenocanthoma—a malignant tumor consisting of both glandular and squamous cell differentiation; rare in CD-1 mice.

Adenocarcinoma—low incidence

(CD-1: none in 891 males, 890 females 6.3%)

(B6C3F₁: none in 2343 males, 2486 females 1.6%)

(B6C3F₁: 2522 females 0.6%).

Fibroadenoma—rare; none in CD-1 mice (B6C3F₁: 3 in 2522 females 0.1%).

Myoepithelioma—infrequent in most strains but more common in BALB mice, especially females.

(B6C3F₁: 1 in 2522 females <0.1%)

Mediastinum

Ectopic thyroid tissue

Mesentery

Ectopic pancreatic tissue.

Fat necrosis—localized death of fat tissue that may be accompanied by encompassing fibrosis and mineralization.

Inflammation (peritonitis)—small aggregates of lymphoid cells are common.

Mouth (*see* Oral cavity)

Muscle (*see* Skeletal muscle)

Nose (nasal sinuses)

Degeneration, hyaline—eosinophilic droplets are commonly present in cells of the respiratory epithelium of some strains such as C57BL and B6C3F₁ mice.

Inflammation (rhinitis, sinusitis)—acute and chronic inflammations are common and associated with respiratory tract infections and inhaled foreign materials.

Hyperplasia, lymphoid—common finding in the nasolacrimal duct.

Tumors—very rare; squamous cell carcinoma (CD-1: none in 891 males, 1 in 890 females 0.1%).

Oral cavity (gingiva, nasopharynx, oropharynx, palate, pharynx)

Inflammation—sporadic small focal aggregates of mononuclear inflammatory cells dispersed throughout the subepithelial tissues.

Tumors—rare; squamous cell papilloma (CD-1: 1 in 891 males 0.1%, none in 890 females).

Ovary

Angiectasis (angiectasia)—common in CD-1 mice
 Amyloidosis—common, associated with systemic amyloidosis (*see* Kidney)
 Atrophy—high incidence in aged mice
 Cyst, all types—common in CD-1 mice
 Dilatation of ovarian bursa—most frequent type
 Hemorrhagic—occasional
 Epidermoid—occasional
 Extramedullary hematopoiesis—usually occurs in association with leukemoid reactions and myeloid hyperplasia (*see* Bone marrow); common in CD-1 mice
 Interstitial cell hyperplasia—must be differentiated from relative increase associated with atrophy
 Pigmentation, ceroid (lipofuscin)—common in CD-1 mice
 Tubular hyperplasia

Tumors

Adenoma

(CD-1: 10 in 890 females 1.1%)
 (B6C3F₁: 1 in 246 females 0.4%)

Cystadenoma—low incidence

(CD-1: 1000 females 2.5%)
 (B6C3F₁: 3 in 246 females 1.2%)

Adenoma, tubular (B6C3F₁: 2167 females 0.9%)

Adenocarcinoma, tubular (B6C3F₁: none in 2167 females)

Papilloma (CD-1: 1 in 890 females 0.1%)

Dysgerminoma—very rare, none in CD-1 mice

Granulosa cell tumors (included thecoma and luteoma)

Low incidence (3.2% in CD-1 mice)
 (B6C3F₁: 2 in 2167 females 0.1%)

Granulosa cell tumor

(CD-1: 500 females 1.0%)
 (CD-1: 2 in 890 females 0.2%)

Granulosa cell tumor, malignant

(CD-1: 1 in 500 females 0.2%)
 (CD-1: 1 in 890 females 0.1%)

Luteoma

(CD-1: 500 females 1.8%)
 (CD-1: 6 in 890 females 0.7%)

Theca cell tumor (thecoma)

(CD-1: 1 in 500 females 0.2%)
 (CD-1: 4 in 890 females 0.5%)

Hemangioma (angioma, hemangioendothelioma)—occasional finding in CD-1 and B6C3F₁
 (CD-1: 4 in 890 females 0.5%)

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—occasional finding
 (CD-1: 1 in 890 females 0.1%)

Ovarian yolk sac carcinoma—rare (B6C3F₁: none in 2390 females)

Sertoli cell tumor—rare

Teratoma—very low incidence
 (B6C3F₁: 1 in 246 females 0.4%)
 (B6C3F₁: 41,000 females 0.2%)

Oviducts

Lesions are often associated with changes in ovary or uterus.

Dilatation—occasional finding.

Inflammation—usually associated with ovarian or peritoneal inflammation.

Tumor

Papilloma—very low incidence (CD-1: 1 in 890 females 0.1%)

Leiomyoma—very low incidence (CD-1: 1 in 890 females 0.1%)

Pancreas

Amyloidosis—common site (*see* Kidney).

Arteritis—frequent finding of fibrinoid necrosis and inflammation that can result in hemorrhage.

Atrophy—common focal finding having lobular pattern with various severities and durations, often accompanied by inflammation.

Cystic degeneration—occasional foci that resemble ballooning (cystic) degeneration common in rat livers.

Dilated ducts—sporadic finding of unknown origin.

Fibrosis, interstitial, diffuse—occasional finding in aged mice.

Inflammation (pancreatitis)—frequent in aged mice (CD-1: 2000 males 2.8%, 2000 females 6.7%); small aggregates of lymphoid cells are common; 20% incidence was observed in 274 C57BL/6 female mice; chronic inflammatory cells are often adjacent to blood vessels or ducts and may be associated with focal acinar atrophy; acute necrotizing pancreatitis can result from virus infections, for example, reovirus and encephalomyocarditis virus as well as septicemic infections.

Edema, interstitial, diffuse—common finding in aging mice usually associated with cardiac failure or severe renal disease and as an agonal change.

Tumors—uncommon in mice

Adenoma, acinar cell—very rare

(CD-1: 1 in 891 males 0.1%, none in 890 females)
 (B6C3F₁: none in 2543 males, 1 in 2522 females <0.1%)

Adenocarcinoma, acinar cell—very rare (B6C3F₁: none in 2543 males, none in 2522 females)

Cystadenoma—very rare (B6C3F₁: none in 2543 males, none in 2522 females)

Parathyroid gland

Amyloidosis—common (*see* Kidney); incidence 10%–40% in CD-1 mice.

Cyst—occasionally present.

Hyperplasia—uncommon; bilateral hyperplasia usually accompanies chronic renal disease (CD-1: 2000 males <1%, 2000 females <1%).

Pigmentation, melanin (melanosis)—common in pigmented strains, such as C57BL.

Thymic rests.

Tumors

Adenoma—very rare <1% in mice (CD-1: none in 891 males, 1 in 890 females 0.1%)

Penis

Inflammation—incidence variable; wounds are common.

The ulceration and abscess formation of the perineal region that accompany urethral plugs in obstructive uropathy may involve the penis and preputial glands (*see Urethra*).

Pericardium

Inflammation—associated with inflammation of adjacent cardiac and mediastinal tissues

Tumors—associated with neoplasia of adjacent tissues

Peripheral nerve (peripheral nervous system)

Few lesions are observed in mice.

Degeneration—sporadic finding in aging mice consisting of vacuoles containing cellular debris and a few macrophages.

Demyelination (segmental, Wallerian, distal axonopathy)—degenerative lesions involving the cell body, axon, and myelin sheath (Schwann cells) in various patterns; special stains and neuropathologic techniques are needed.

Tumors—the terminology and classification of peripheral nerve tumors is complex and confusing:

Neurofibroma (perineural fibroma)—rare.

Neurofibrosarcomas are reported in skin and large intestine of B6C3F₁ mice, <0.1%.

Schwannoma, benign or malignant (neurinoma, neurilemmoma, neurolemma, neurofibroma, neurofibrosarcoma)—some of these tumors were previously termed reticulum cell sarcoma, type A; sites of predilection: uterus, epididymis, and spinal nerve roots.

Ganglioneuroma—rare tumor, more often observed in the adrenal gland than the spinal ganglia.

Peritoneum

Extramedullary hematopoiesis—usually occurs in the perirenal fat associated with leukemoid reactions and myeloid hyperplasia (*see Bone marrow*)

Inflammation—associated with inflammation of adjacent abdominal organs and tissues

Necrosis, fat (B6C3F₁: 2543 males 1.3%, 2522 females 1.1%)

Tumors—most often are secondary and associated with neoplasia of abdominal organs

Mesothelioma (mesenchymal sarcoma)

(CD-1: none in 891 males, 1 in 890 females 0.1%)

(B6C3F₁: 3 in 2343 males 0.1%, and none in 2486 females)

Pharynx (*see Oral cavity*)

Pituitary gland

Cyst, simple—common; usually in pars distalis

Extramedullary hematopoiesis—usually occurs in association with leukemoid reactions and myeloid hyperplasia (*see Bone marrow*)

Hyperplasia, focal—low incidence, 1%–2% CD-1 mice (CD-1: 2000 males <1%, 2000 females <1%)

Tumors

Adenoma, pars distalis—low incidence

(RF: 427 females 1.4%)

(CD-1: 2 in 891 males 0.2%, 890 females 4.7%)

(CD-1: 2000 males <1%, 2000 females 1%)

(B6C3F₁: 1903 males 0.6%, 2051 females 7.9%)

Carcinoma (adenocarcinoma), pars distalis

(CD-1: 2 in 891 males 0.2%, 2 in 890 females 0.2%)

(CD-1: 2000 males none, 2000 females <1%)

(B6C3F₁: 1 in 1903 males 0.1%, 2051 females 0.4%)

Pleura

Usually pleural lesions are the result of changes in the underlying lung or adjacent mediastinum and thorax; neoplastic lesions usually result from systemic or multifocal tumors in the lungs.

Tumors

Mesothelioma (mesenchymal sarcoma)—very rare (CD-1: one reported in an untreated female).

Alveolar/bronchiolar (adeno-)carcinomas (typically) and mammary gland (adeno-)carcinomas (less often) invade the pleura from the lungs.

Malignant fibrous histiocytosis, malignant histiocytoma, and malignant lymphoma—occasionally involve the pleura.

Preputial gland

Atrophy—minor degrees are common in aged mice.

Cysts—minor degrees are common in aged mice.

Enlargement—frequently observed grossly.

Hyperplasia, squamous cells of duct epithelium—often accompanies suppurative inflammation or abscess.

Inflammation (adenitis): acute and chronic—common (up to 10%, CD-1 mice); often results after wounds from fighting; ulceration and abscess formation of the perineal region including the preputial glands accompany urethral plugs in obstructive uropathy (*see Urethra*).

Tumors

Adenoma—uncommon finding

(CD-1: 1 in 891 males 0.1%)

(B6C3F₁: 2 in 2343 males 0.1%)

Carcinoma—very rare (B6C3F₁: 1 in 2855 males <0.1%)

Myoepithelioma—infrequent in most strains but more common in BALB mice

Prostate

Atrophy—associated with simple squamous epithelium and dilatation

Dilatation (ectasis)—usually associated with atrophy

Hyperplasia, epithelial—occasional

Inflammation (prostatitis)

Chronic—frequently present in interstitial tissues

Suppurative—usually associated with inflammation of seminal vesicles, coagulation glands, urinary bladder, or kidneys (pyelonephritis)

Tumors

Adenoma—none in CD-1 mice

Adenocarcinoma—very rare (CD-1: 1 in 891 males 0.1%)

Salivary gland (sublingual, mucous; parotid, serous; submandibular or submaxillary, mixed)

Atrophy—occasional finding, usually lobular distribution in CD-1 mice.

Hyperplasia, lymphoid—aggregates of lymphoid cells tend to accumulate around ducts.

Inflammation (adenitis)—common finding of small foci of lymphocytes and plasmocytes.

Tumors

Adenoma—uncommon finding

Adenoma, parotid gland (CD-1: none in 891 males, 2 in 890 females 0.2%)

Adenoma, submaxillary gland (CD-1: 2 in 891 males 0.2%, none in 890 females)

Adenocarcinoma—very rare; none reported in more than 10,000 CD-1 and B6C3F₁ mice

Myoepithelioma—infrequent in most strains but more common in BALB mice, especially females

Seminal vesicles

Dilatation, cystic—common

Inflammation

Chronic—common in interstitium

Suppurative—usually associated with prostate, urinary bladder, or kidney inflammation (pyelonephritis)

Tumors

Adenoma—uncommon finding (CD-1: 1 in 891 males 0.1%)

Adenocarcinoma—uncommon finding (CD-1: 1 in 891 males 0.1%)

Granular cell tumor—very rare

Skeletal muscle

Atrophy—occasional focal finding in aged mice

Inflammation, chronic (myositis)—sporadic foci of lymphoid cells in CD-1 mice

Tumors

Rhabdomyosarcoma—very rare in mice (B6C3F₁: 2343 males 0.5%, 2 in 2486 females 0.1%)

Skin

Amyloidosis—low incidence, usually with systemic amyloidosis (*see* Kidney).

Epidermoid cyst—low incidence.

Hair loss (alopecia)—incidence variable, frequently physiological, but may be due to a variety of causes, including hair chewing or vices, genetics, mites, and local or systemic disease; patchy hair loss is common.

Inflammation (dermatitis)—incidence variable, frequently present; especially associated with fighting and wounds of ears, feet, penis, and tail.

Tumors, epidermal

Basal cell tumors, all types (B6C3F₁: 2 in 2343 males 0.1%, 2486 females 0.2%)

Basal cell carcinoma—low incidence <1% CD-1 (CD-1: none in 891 males, 1 in 890 females 0.1%)

Keratoacanthoma—low incidence

(CD-1: 1 in 891 males 0.1%, 1 in 890 females 0.1%) (B6C3F₁: 2343 males none, 2486 females none)

Lymphoma—incidence variable, usually associated with multiple organ involvement

Sebaceous adenoma—very low incidence

Sebaceous carcinoma—very low incidence

Squamous cell papilloma—very low incidence CD-1

(CD-1: 2 in 891 males 0.2%, none in 890 females) (B6C3F₁: 3 in 2343 males 0.1%, 2486 females 0.2%)

Squamous cell carcinoma—very low incidence

(CD-1: 891 males 0.2%, 890 females 0.7%) (B6C3F₁: 2343 males 0.2%, 2486 females 0.2%)

Tumors, subcutaneous tissue

Angioma (hemangioma, hemangioendothelioma)—low incidence CD-1 (CD-1: 891 males 0.5%, 890 females 0.9%)

Angiosarcoma (hemangiosarcoma, hemangioendotheliosarcoma)—very low incidence CD-1 (CD-1: 891 males 0.3%, 890 females 0.8%)

Fibroma—very low incidence <1% CD-1 (CD-1: 1 in 891 males 0.1%, 3 in 890 females 0.3%)

(Neuro-)fibroma (B6C3F₁: 2343 males 1.2%, 1 in 2486 females <0.1%)

Fibrosarcoma—low incidence <2% CD-1 (CD-1: 891 males 1.7%, 890 females 0.9%)

(Neuro-)fibrosarcoma (B6C3F₁: 2343 males 2.8%, 2486 females 0.8%)

Fibrous histiocytoma—low incidence 4% CD-1 (CD-1: 891 males 0.3%, 890 females 0.5%)

Leiomyoma—none in CD-1

Leiomyosarcoma (CD-1: none in 891 males, 1 in 890 females 0.1%)

Lipoma—very low incidence CD-1

Liposarcoma—very low incidence CD-1 (CD-1: none in 891 males, 890 females 0.8%)

Sarcoma, undifferentiated (sarcoma, NOS)—very low incidence CD-1 (B6C3F₁: 2343 males 1.7%, 2486 females 0.7%)

Spinal cord (*see* Brain)

Neuroaxonal dystrophy—occasional finding in which terminal axons, especially in the nucleus gracilis, are swollen in aged mice

Squamous epithelial cyst—uncommon finding

Tumors—rare (*see* Brain)

Spleen

Amyloidosis—frequent in CD-1 mice (*see* Kidney) (CD-1: 2000 males 11.6%, 2000 females 11.6%).

Atrophy—lymphocyte depletion is common in aged mice.

Extramedullary hematopoiesis—minimal to moderate degrees are considered to be normal; marked or severe degree is abnormal; usually occurs associated

with leukemoid reactions and myeloid hyperplasia (*see* Bone marrow) (CD-1: 2000 males 7.6%, 2000 females 7.6%).

Fibrosis—rare.

Hyperplasia, erythroid—increased erythroid precursors are associated with anemia and extensive neoplasia.

Hyperplasia, lymphoid—low incidence, usually in females (C57BL/6: 274 female mice 5%)

(CD-1: 2000 males 13.9%, 2000 females 13.9%).

Hyperplasia, megakaryocytic—usually accompanies myeloid hyperplasia.

Hyperplasia, myeloid (leukemoid reaction)—increased immature granulocytic precursors associated with systemic inflammatory conditions (*see* Bone marrow).

Pigmentation, hemosiderin—hemosiderin is normal in moderate numbers of macrophages in mice; increased hemosiderin is associated with some forms of anemia, hemolysis, and increased erythrocyte destruction.

Pigmentation, lipofuscin—occurs in some strains.

Pigmentation, melanin (melanosis)—commonly affects the splenic capsule and trabeculae in pigmented strains, such as C57BL; must be differentiated from hemosiderin.

Tumors

Hemangioma (angioma, hemangioendothelioma)

(CD-1: 891 males 0.2%, 890 females 0.1%)

(CD-1: 2000 males 0.4%, 2000 females 0.4%)

(B6C3F₁: 3766 males 0.1%, 3751 females 0.2%)

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)

(CD-1: 2000 males 0.6%, 2000 females 0.6%)

(B6C3F₁: 3766 males 2.1%, 3751 females 1.0%)

Histiocytic sarcoma (reticulum cell sarcoma, type A)—rare before 12 months; common especially after 18 months of age; frequently involves the uterus, vagina, and liver and less often the bone marrow, lungs, pancreas, dermis, mesentery, kidney, and epididymis (RF: reticulum cell sarcoma, 427 females 56.2%)

(CD-1: 2000 males 1.2%, 2000 females 1.2%)

Leukemia, myelogenous (myeloid, granulocytic)—often primarily involves liver and spleen in RF strain; must be differentiated from leukemoid reaction and myeloid hyperplasia

(CD-1: 2000 males 1.9%, 2000 females 1.9%)

(B6C3F₁: systemic, 2343 males 0.7%; 2486 females 2.1%)

Leukemia, lymphatic (lymphocytic, lymphoblastic)—malignant lymphomas that have disseminated into the peripheral blood

Lymphoid tumors—frequently involve the spleen (*see* Lymph node)

(RF: thymic lymphoma, 427 females 4.0%)

(RF: nonthymic lymphoma, 427 females 7.0%)

Malignant lymphoma (lymphosarcoma), all types

(C57BL/6: 2 in 274 female mice 0.7%)

(CD-1: 2000 males 5.3%, 2000 females 5.3%)

Mast cell tumor, malignant (B6C3F₁: 1 in 3766 males <0.1%, none in 3751 females)

Plasma cell tumor, malignant (B6C3F₁: none in 3766 males, 1 in 3751 females <0.1%)

Stomach (glandular)

Amyloidosis—common; begins basally (*see* Kidney).

Arteritis—sporadic finding.

Cysts—very common to have intramucosal cysts; when large cyst walls often undergo squamous metaplasia.

Erosion (*see* Ulcer) (CD 1: 2000 males <1%, 2000 females <1%).

Degeneration, hyaline—eosinophilic droplets are commonly present in epithelial cells of aged B6C3F₁ mice.

Diverticulum—rare finding.

Gastric hepatocytes—uncommon lesions in CD-1 and B6C3F₁ aged mice.

Glandular hyperplasia (adenomatous hyperplasia, hypertrophic gastritis, proliferative gastritis)—frequent finding, up to 20% of CD-1 mice; lower in B6C3F₁; occasional finding of glandular elements in the submucosa

(CD-1: 2000 males 2.6%, 2000 females 2.8%)

(B6C3F₁: 2543 males 0.8%, 2522 females 0.7%).

Inflammation (gastritis)—acute and chronic active inflammations are common findings, often accompanying erosions or ulcerations; scattered inflammatory cells can occur in the mucosa without ulceration or necrosis; occasionally crypts contain leukocytes (crypt abscess); chronic inflammation with lymphoid cells is occasionally observed in the submucosa:

(CD-1: 2000 males 2%, 2000 females 2%)

(B6C3F₁: 2543 males 1.8%, 2522 females 1.9%).

Mineralization (calcification)—focal aggregates of calcium are found in the gastric glandular epithelium; metastatic calcification occurs in the glandular mucosa in association with severe renal disease and parathyroid hyperplasia; dystrophic calcification also occurs in the smooth muscles:

(CD-1: 2000 males <1%, 2000 females <1%)

Necrosis—superficial necrosis (erosion) and deep necrosis (ulceration) are occasional findings (*see* Ulcer) (CD-1: 2000 males 1%, 2000 females <1%).

Ulcer—erosions and ulcers (focal necrosis) are sporadic low incidence findings that can result from a variety of causes accompanied by acute or chronic active inflammation (acute or chronic necrotizing inflammation) (CD-1: 2000 males <1%, 2000 females <1%).

Tumors—rare in CD-1 and B6C3F₁ mice

Adenoma—low incidence

(CD-1: 1 in 891 males 0.1%, none in 890 females)

(B6C3F₁: 1 in 3807 males <0.1%, 1 in 3789 females <0.1%)

Adenocarcinoma—low incidence, <0.1%

(B6C3F₁: 1 in 2543 males <0.1%, 1 in 2522 females <0.1%)

(B6C3F₁; 1 in 3807 males <0.1%, 1 in 3789 females <0.1%)

Adenomatous polyp—very low incidence (B6C3F₁; 2 in 2543 males <0.1%, 1 in 2522 females <0.1%)

Neuroendocrine cell tumors (gastric carcinoid, APUDoma)—rare in mice (B6C3F₁; 1, malignant, in 3807 males <0.1%; 1, malignant, in 3789 females <0.1%)

Stomach (nonglandular) (*see* Forestomach)

Subcutaneous tissue (*see* Skin)

Testes

Atrophy of seminiferous tubules—common finding; occurs in a variety of patterns and frequencies; may be associated with a relative increase in Sertoli cell numbers (CD-1: 2000 males 2.7%).

Degeneration of seminiferous tubules—low incidence (B6C3F₁; 2543 males 1.5%).

Edema—must be differentiated from a common artifact resembling central edema.

Inflammation, spermatic granulomas—occasional.

Interstitial (Leydig) cell hyperplasia—diffuse hyperplasia, commonly accompanies atrophy; focal hyperplasia must be differentiated from adenoma.

Mineralization, focal—common.

Periarteritis—occasional

Fibrinoid arteriopathy—occasional

Amyloidosis—occasional (*see* Kidney)

Tumors

Embryonal carcinoma—rare (B6C3F₁; 2 in 2543 males <0.1%)

Interstitial (Leydig) cell tumors, all types

(CD-1: 8 in 891 males 0.9%)

(CD-1: 2000 males <1%)

(B6C3F₁; 2543 males 0.3%)

Interstitial (Leydig) cell adenoma—low incidence, 1%–4% in CD-1 mice; less in B6C3F₁

Interstitial (Leydig) cell carcinoma—very rare

Papillary adenoma (CD-1: 1 in 891 males 0.1%)

Seminoma—very rare

(B6C3F₁; 1 in 244 males 0.4%)

(B6C3F₁; 1 in 2543 males <0.1%)

Sertoli cell tumor—very rare

Stromal tumor (CD-1: 1 in 891 males 0.1%)

Teratoma—very rare

Thymus

Amyloidosis (*see* Kidney) (CD-1: 2000 males 0.4%, 2000 females 0.4%).

Atrophy—normal involution begins at sexual maturity; atrophy is associated with stress and infections: (CD-1: 2000 males 1.7%, 2000 females 1.7%)

Cyst—frequent finding in both cortex and medulla (CD-1: 2000 males 0.6%, 2000 females 0.6%).

Ectopic parathyroid tissue—sporadic finding.

Hyperplasia, lymphoid—frequent finding especially in female mice after 6 months of age (CD-1: 2000 males 19%, 2000 females 19%).

Tumors

Histiocytic sarcoma (reticulum cell sarcoma, type A)—(CD-1: 2000 males 0.9%, 2000 females 0.9%).

Leukemia, myelogenous (myeloid, granulocytic) (CD-1: 2000 males 1.4%, 2000 females 1.4%).

Malignant lymphoma (lymphosarcoma), all types—frequently involves the thymus

(CD-1: 2000 males 0.4%, 2000 females 0.4%)

(CD-1: 891 males 0.6%, 890 females 0.6%).

Thymic lymphomas reportedly arise in the thymus of some strains of mice such as RF and then predominantly involve the mediastinal organs, lungs, and heart, with subsequent spread to other organs, including the liver, spleen, ovaries, uterus, and peripheral lymph nodes (also *see* Lymph node).

Thymoma—rare tumors composed of a mixture of epithelial and lymphoid elements

(CD-1: 2000 males <0.1%, 2000 females <0.1%)

(CD-1: 1 in 891 males 0.1%, none in 890 females).

Thyroid gland

Amyloidosis—common; incidence 10%–40% CD-1 mice (*see* Kidney)

Cyst—common; often lined by ciliated or squamous cells; pharyngobranchial duct remnant (ciliated lining); ultimobranchial duct remnant (squamous lining)

Thymic rests

Hyperplasia, C cell (CD-1: 2000 males <0.1%, 2000 females <0.1%)

Hyperplasia, follicular cell

Tumors

Adenoma, C cell—very low incidence

(CD-1: 2000 males <0.1%, 2000 females <0.1%)

(B6C3F₁; none in 2178 males, 2 in 2203 females 0.1%)

Adenocarcinoma, C cell—very low incidence

(CD-1: 2000 males <0.1%, none in 2000 females)

(B6C3F₁; none in 2178 males, none in 2203 females)

Adenoma, follicular cell—very low incidence, 1% or less in CD-1 mice

(CD-1: 891 males 0.9%, 890 females 0.7%)

(B6C3F₁; 2178 males 1%, 2203 females 1.8%)

Adenocarcinoma, follicular cell (B6C3F₁; 2178 males 0.2%, 2203 females 0.3%)

Tongue

Inflammation—sporadic; often associated with mineralization

Mineralization—foci of calcification in muscles adjacent to lamina propria with concurrent granulomatous inflammation (*see* Heart)

Tumors—rare; squamous cell carcinomas reported in B6C3F₁

Tooth

Dysplasia

Malocclusion—acquired and genetic predisposition

Trachea

Calcification—occasional finding in tracheal cartilage

Inflammation, acute and chronic (tracheitis)—frequent finding of small foci of inflammatory cell infiltrates; low incidence (up to 10%, CD-1 mice), sporadic finding resulting from infections caused by gavage accidents

Osseous metaplasia—occasional finding in tracheal cartilage

Tumors—none reported in CD-1 mice

Urinary bladder

Inflammation, acute and chronic (cystitis)—aggregates of lymphoid cells are common, especially in the submucosa; approximately 10% of CD-1 mice; more severe inflammation is usually associated with inflammation of the prostate or pyelonephritis (CD-1: 2000 males 6.1%, 2000 females 12.9%).

Dilatation—frequently observed at necropsy; important only in obstructive uropathy (*see* Urethra).

Hyperplasia, urothelial (epithelial)—uncommon; usually associated with cystitis or calculi (CD-1: none in 2000 males, 2000 females <0.1%).

Tumors

Transitional cell papilloma—very rare in mice (B6C3F₁: 2 in 3807 males <0.1%, none in 3789 females)

Transitional cell carcinoma—very rare in mice (CD-1: 1 in 891 males 0.1%, none in 890 females)

Ureter (*see* Kidney)

Urethra

Mucous plugs—common.

Obstructive uropathy (urologic syndrome, obstructive genitourinary disease, dysuria)—a condition affecting male mice with a high mortality rate, 30%–40%, which is often associated with mucous plugs, and urinary bladders distended with urine.

Uterus

Angiectasis, myometrial—common.

Adenomyosis—low incidence (1%–3%, CD-1 mice).

Hyperplasia, cystic endometrial—very common (>50% of CD-1 mice).

Hyperplasia, stromal cell—usually associated with cystic endometrial hyperplasia.

Hydrometra—common.

Metaplasia, squamous—usually associated with pyometra.

Mucometra—common finding characterized by dilatation of the uterine horns containing excessive mucinous contents; small amounts are normal during parts of the estrus cycle.

Pyometra (suppurative inflammation)—occasional.

Tumors

Adenoma—low incidence (CD-1: 5 in 890 females 0.6%)

Adenocarcinoma—very low incidence (CD-1: 2 in 890 females 0.2%)

Choriocarcinoma—very rare

Hemangioma (angioma, hemangioendothelioma)—low incidence, 1%–2% in CD-1 mice (CD-1: 8 in 890 females 0.9%)

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—occasional finding (CD-1: 1 in 890 females 0.1%)

Leiomyoma—low incidence, 1%–2% in CD-1 mice (CD-1: 21 in 890 females 2.4%)

Leiomyosarcoma—low incidence, 1%–2% in CD-1 mice (CD-1: 12 in 890 females 1.4%)

Papilloma (CD-1: 5 in 890 females 0.6%)

Uterine stromal cell polyp—low incidence (B6C3F₁: 2360 females 0.9%)Uterine stromal cell sarcoma (endometrial sarcoma)—low incidence, 2%, CD-1 mice (CD-1: 2 in 890 females 0.2%) (B6C3F₁: 2360 females 0.6%)Yolk sac carcinoma—very rare (none in CD-1 mice) (B6C3F₁: none in 2445 females)

Vagina

Inflammation—usually associated with changes of the uterus

Tumors

Adenocarcinoma—uncommon finding (B6C3F₁: 1 in 246 females 0.4%)

Leiomyosarcoma—uncommon finding (CD-1: 1 in 890 females 0.1%)

Zymbal's gland (*see* Ear)**HISTOPATHOLOGIC FINDINGS IN CONTROL LABORATORY RATS^{10,11,13,18,19,21–25,27–29,33–47}**

Adrenal gland, cortex

Accessory cortical nodule—ancillary cortical tissue surrounded by a fibrous capsule.

Amyloidosis—very rare observation in Fischer 344 rats.

Angiectasis (telangiectasis)—common (Sprague–Dawley; 2000 males 4.6%, 2000 females 28.3%).

Atrophy, brown (pigmentation, brown degeneration, ceroid deposition, hemosiderin)—uncommon in rat; usually associated with severe diffuse atrophy or focal compression by space-occupying lesion.

Atrophy, diffuse—usually related to increased adrenocortical steroids, uncommon finding.

Congestion—common finding; usually incidental.

Cortical changes, focal (cytoplasmic alterations)

Basophilic cell foci—commonly seen in zona fasciculata of aged rats

Clear cell foci—commonly seen in zona glomerulosa of aged rats

Eosinophilic cell foci—commonly seen in zona fasciculata of aged rats

Mixed cell foci—commonly seen in zona glomerulosa of aged rats

Cystic degeneration (cystic change, peliosis)—similar to cystic change in the liver, especially reported in old breeder female rats; appear to start as foci of large eosinophilic cells (focal cortical hypertrophy) that degenerate to form cysts (Sprague–Dawley: 578 males 23.4%, 585 females 82.7%).

Extramedullary hematopoiesis—occasional finding.

Fatty change, diffuse—commonly seen as cytoplasmic vacuolation of the adrenal cortical cells.

Hemosiderosis—small amounts of iron-positive pigment can be observed occasionally in the deep cortex.

Hemorrhage—common finding; usually incidental or accompanying cystic degeneration, inflammation, or necrosis.

Hyperplasia, diffuse (lipid depletion)—lipid depletion occurs under various conditions; primarily affects zona fasciculata characterized by cells with dense eosinophilic cytoplasm; may be associated with increased adrenal weight.

Hyperplasia, focal—common finding (Sprague–Dawley: 578 males 11.1%, 585 females 12.0%).

Inflammation, focal, acute—occasionally nonspecific accompaniment to several generalized inflammatory diseases.

Necrosis, focal—common finding; often accompanying cystic degeneration or inflammation.

Osseous metaplasia—rare finding in Fischer 344 rat.

Pigmentation (*see* Atrophy, brown).

Tumors

Cortical adenoma—low incidence

(Fischer 344/N: 1915 males 1.2%, 1968 females 2.8%)

(Sprague–Dawley: 578 males 1.6%, 585 females 4.6%)

Cortical carcinoma (adenocarcinoma)—low incidence

(Fischer 344/N: 1915 males 0.1%, 1968 females 0.2%)

(Sprague–Dawley: 578 males 1.0%, 585 females 0.5%)

Adrenal gland, medulla

Hyperplasia, focal—small foci are common that must be differentiated from pheochromocytomas (Sprague–Dawley: 578 males 30.3%, 585 females 16.9%).

Hypertrophy.

Tumors

Ganglioneuroma—very low incidence (Fischer 344/N: 1915 males 0.3%, 1968 females 0.2%)

Pheochromocytoma, all types—very common, especially in Long–Evans strain, and less in Sprague–Dawley (Fischer 344/N: 1915 males 25.5%, 1968 females 5%)

Pheochromocytoma, benign (Sprague–Dawley: 578 males 19.0%, 585 females 5.3%)

Pheochromocytoma, malignant (Sprague–Dawley: 578 males 1.9%, 585 females 0.9%)

Blood vessels

Angiectasis (ectasia)—common finding in endocrine organs, especially pituitary and adrenal glands with hyperplasia and neoplasia, less frequent in the ovaries.

Arteriosclerosis—a variety of nonspecific changes occur in arteries including intimal plaque formation, medial degeneration (mucoid degeneration), hyaline degeneration, medial hypertrophy, and mineralization (calcification); involves aorta, iliac, renal, carotid, and cerebral arteries; incidence and severity appear to vary with strain, not reported in Fischer 344 rats.

Arteritis (polyarteritis nodosa, periarteritis, perivascularitis)—frequent finding, most often in medium arteries of the testes, pancreas, mesentery, and occasionally the heart, ovary, uterus, brain, adrenal, and liver; characterized by inflammatory infiltrates of all layers of the vessel wall and necrosis (fibrinoid necrosis, necrotizing inflammation); incidence may be reduced by food restriction; incidence influenced by strain

(Fischer 344/N: males 1.8%, females 0.9%)

(Long–Evans: males 4.5%, females 2.6%)

(Wistar: males 9.1%, females 4.2%)

(August: males 45.4%, females 43.0%).

Atherosclerosis is not reported as a spontaneous lesion in laboratory rats.

Hemorrhage—occasional spontaneous finding in several organs, particularly the thymus, lymph nodes, and lungs; often agonal change or associated with necropsy technique.

Hypertrophy, medial—major pulmonary arteries of the aged Sprague–Dawley rat frequently have medial hypertrophy; other strains appear to have a low incidence.

Mineralization—commonly observed associated with arteriosclerosis; isolated subendothelial deposits of calcium salts are sometimes seen in the pulmonary arteries of aged rats.

Thrombosis—occasional finding in the pulmonary and hepatic veins.

Tumors

Hemangioma (angioma, hemangioendothelioma), all sites—very low incidence (Fischer 344/N: 2 reported in 1936 males 0.1%, 3 reported in 1983 females 0.2%)

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma), all sites—low incidence (Fischer 344/N: 1936 males 0.5%, 1983 females 0.3%)

Hemangiopericytoma—very rare (Fischer 344/N: less than 10 in 50,000 rats <0.1%)

Paraganglioma, aortic body—rare in most strains, except female WAG/Rij rat 12%

Paraganglioma, retroperitoneal (near kidney) (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)

Bone

Cysts—solitary bone cysts are observed sporadically.

Degeneration, mucinous or chondromucinous, cystic—a common lesion of cartilage, especially in the sternum

but also other sites involving articular and growth plate cartilage.

Exostosis—hyperplasia associated with trauma, fractures, degenerative disease, and chronic infections.

Fracture callus—common in ribs and extremities, especially feet and tail from restraining or fighting.

Inflammation (osteomyelitis)—most common in bones of jaw, legs, feet, and tail associated with trauma or spread of infections from adjacent abscesses or ulcerated tumors.

Necrosis, aseptic—also termed mucinous degeneration; may occur in the ossified menisci as ghostlike areas of bone.

Osteodystrophy, fibrous (osteitis fibrosis, osteitis fibrosa)—sporadic finding associated with hyperparathyroidism and severe renal disease; unusually high in OFA-ICO strain of rat.

Osteochondrosis—developmental defect of endochondral ossification involving the femur and humerus reported in Sprague-Dawley.

Osteophytes (chondroosseous outgrowths, spondylosis)—observed on vertebrae, primarily the ventral aspect in males; reported in Fischer 344 and other strains.

Osteopetrosis (osteosclerosis, hyperostosis)—thickening of trabeculae or plates of bone, most often detected in calvaria of skull, nasal turbinates, and spongy bone of femur.

Tumors—occasional finding especially osteosarcomas

Chordoma—uncommon malignant neoplasm arising in the vertebral column from embryonic notochord

Osteoma, all sites—very low incidence (Fischer 344/N: 2 reported in 1949 males 0.1%)

Osteosarcoma, all sites—very low incidence (Fischer 344/N: 1936 males 0.4%, 1983 females 0.4%)

Chondrosarcoma, all sites—very low incidence (Fischer 344/N: 1 reported in 1949 males 0.1%, 1 reported in 1950 females 0.1%)

Hemangiosarcoma, vertebra (Fischer 344/N: 1 reported in 1936 males 0.1%)

Hemangiosarcoma, pelvis (Fischer 344/N: 1 reported in 1983 females 0.1%)

Bone marrow

Atrophy (hypoplasia, aplasia, panmyelopathy)—sporadic finding in aged rats in which hematopoietic cells are replaced fat (adipose) cells.

Fibrosis—occasional small foci in the marrow space.

Hyperplasia—the bone marrow of the rat is normally very cellular and with a few scattered fat cells fills the marrow cavity; under various stimuli, the marrow reacts by completely filling the cavity with normal hematopoietic cells; the most common responses consist predominantly of erythroid and myeloid cells; less commonly eosinophils, basophils, or megakaryocytes.

Hyperplasia, erythroid—associated with anemia and increased red cell destruction.

Hyperplasia, histiocytic, focal—proliferation of histiocytes in foci surrounded by inflammatory cells.

Hyperplasia, megakaryocytic—associated with increased platelet consumption.

Hyperplasia, myeloid (leukemoid reaction)—associated with neutrophilia in the peripheral blood; severe myeloid hyperplasia that commonly accompanies extensive necrosis or systemic infections must be differentiated from leukemia.

Hyperplasia, plasma cell—commonly observed; may be accompanied by increased numbers of mast cells.

Hyperplasia, stromal cell—occasional small focal lesions of adventitial reticular cells.

Inflammation, granulomatous—occasional focal lesions observed in young adult and aged rats characterized by aggregates of macrophages or histiocytes; may be same as lesion termed histiocytic hyperplasia.

Tumors

Myelogenous leukemia (granulocytic leukemia)—(Sprague-Dawley: 585 males 0.3%, 585 females 0.2%).

Mononuclear cell leukemia (large granular lymphocytic leukemia, monocytic leukemia, lymphosarcoma)—appears to arise in the spleen and secondarily involves the bone marrow and lymph nodes; common in Fischer 344 rats (Fischer 344/N: 1936 males 33.6%, 1983 females 20.2%); incidence is reportedly decreased in males by gavage with corn oil vehicle (Fischer 344/N: 1949 males 17.1%, 1950 females 19.3%).

Brain and meninges (CNS)

Atrophy, focal (compression)—common finding in rats with expanding pituitary adenomas, particularly if more than 0.5 cm in diameter; commonly associated with hydrocephalus.

Chromatolysis—occasional finding characterized by dispersion and dissolution of Nissl substance.

Dark cells—common finding; foci of neurons that appear shrunken and basophilic without changes in the surrounding parenchyma or glial cell are regarded as artifacts.

Edema—sometimes associated with tumors, especially pituitary adenomas.

Gitter cells—microglial macrophages usually associated with myelin breakdown.

Hemorrhage—common finding occurring from various causes, such as vascular changes associated with pituitary adenomas, leukemia, gliomas, and infarcts.

Hydrocephalus—uncommon finding sometimes associated with compression by large pituitary adenomas.

Infarction—uncommon focal finding as a result of vascular blockage, usually observed as a healing area of chronic inflammation with lipid and hemosiderin in macrophages.

Inflammation—uncommon in laboratory rats unless extensive bacterial respiratory infections are present; occasional finding of small foci of chronic inflammatory cells, lymphocytes, and plasma cells in the brain and meninges

or spontaneous arteritis of the cerebral arteries; chronic encephalitis associated with *Toxoplasma gondii* or focal granulomatous inflammation in the absence of necrosis associated with *Encephalitozoon (Nosema) cuniculi* may be observed in apparently normal rats.

Malacia—necrosis of neural tissues with loss of architecture, breakdown of myelin or neuropil, and accumulation of foamy macrophages (Gitter cells).

Mineralization, cerebral—common, rounded, or irregular basophilic bodies or concretions are found occasionally in rat brains but sometimes reach incidences of up to 20%.

Necrosis—ischemic neuronal injury, occasional finding of shrunken, hyaline, densely eosinophilic cell bodies and small, basophilic nuclei that results from a variety of causes including ischemia, anoxia, seizures, epilepsy, and some metabolic disturbances (*see* Malacia).

Pigmentation

Lipofuscin (ceroid pigment)—periodic acid–Schiff (PAS) reagent positive and acid-fast pigment is found in cerebral neurons with increasing age.

Hemosiderin (Perl's positive iron pigment)—found in macrophages associated with focal infarcts, hemorrhage, inflammation, or around expanding neoplasms.

Melanin—commonly seen in the meninges and the pituitary gland of pigmented strains of rats of all ages.

Vacuolation (vacuolar degeneration, vacuolar encephalopathy)—vacuoles are a frequent finding associated with a variety of causes including artifacts, intramyelinic edema, spongy degeneration, spongiform encephalopathies, uremia, and increasing age.

Tumors

CNS tumors are uncommon in most rats; incidences at 26 months of age were males 1.0% and females 0.8% and at 34 months in life-span studies were males 2.9% and females 2.2%.

Astrocytoma (astrocytic glioma, glioblastoma multiform)—low incidence (Fischer 344/N: 1928 males 0.5%, 1969 females 0.9%) (Sprague–Dawley: 585 males 0.7%, 585 females 0.5%)

Ependymoma—very low incidence (Fischer 344/N: 2 reported in 1943 males 0.1%, 1 reported in 1946 females 0.1%).

Ependymoblastoma—very low incidence (Sprague–Dawley: 1 reported in 585 males 0.2%).

Glioma, all types (Fischer 344/N: 2 reported in 1928 males 0.1%, 1 reported in 1969 females 0.1%).

Glioma, malignant (Sprague–Dawley: 1 reported in 585 males 0.2%).

Granular cell tumors—common in cranial cavity of several rat strains; most are benign; very low incidence (Fischer 344/N: 1928 males 0.2%, 1946 females 0.3%) (Sprague–Dawley: 1 reported in 585 males 0.2%).

Hemangioma (angioma, hemangioendothelioma) (Fischer 344/N: 1 reported in 1969 females 0.1%).

Lymphoreticular tumors (lymphoma, malignant reticulosis, lymphoreticulosis, reticulum cell sarcoma,

microglioma, gliomatosis)—uncommon mesenchymal tumors with unclear relationship to undifferentiated gliomas.

Medulloblastoma—very low incidence

(Fischer 344/N: 1 reported in 1928 males 0.1%, 1 reported in 1969 females 0.1%)

(Sprague–Dawley: 1 reported in 585 males 0.2%)

Meningioma—commonest tumor in the CNS of some rat colonies with syncytial and fibroblastic types; very low incidence (Fischer 344/N: 2 reported in 1928 males 0.1%, 2 reported in 1969 females 0.1%).

Oligodendroglioma—very low incidence

(Fischer 344/N: 2 reported in 1928 males 0.1%, 3 reported in 1969 females 0.2%)

(Sprague–Dawley: 1 reported in 585 males 0.2%)

Clitoral gland

Atrophy—occasional finding involving the acinar epithelium in aging rats.

Cyst—minor cystic changes commonly accompany atrophy.

Degeneration, cystic—common finding in aged males; combination of changes including atrophy of acinar cells, fibrosis, distention of ducts by secreted material, and limited degrees of inflammation.

Enlargement—frequently observed grossly.

Hyperplasia, focal—occasional finding of the acinar epithelium in aging rats; sometimes cystic.

Hyperplasia, squamous cells of duct epithelium—often accompanies suppurative inflammation or abscess.

Inflammation (adenitis)—occasional finding, especially suppurative inflammation or abscesses.

Tumors—usually arise from acinar or ductal epithelium

Adenoma—low incidence (Fischer 344/N: 1983 females 2.8%)

Carcinoma—low incidence (Fischer 344/N: 1983 females 3.0%)

Squamous cell papilloma (Fischer 344/N: 1 reported in 1950 females 0.1%)

Coagulating gland

Inflammation, chronic—common incidental focal finding of lymphocytic infiltrations

Inflammation, suppurative—usually associated with prostatic, urinary bladder, or kidney inflammation

Tumors—none reported in 51,230 Fischer 344/N rats

Ductus (vas) deferens (*see* Epididymis)

Ear

External ear (pinna, auricle) and auditory canal

Auricular chondropathy (proliferative chondritis)—nodular lesions characterized by granulomatous inflammation and chondrolysis with regenerative hyperplasia and fibrosis; occurrence is strain related; most frequent in Sprague–Dawley and fawn-hooded rats; not observed in Fischer 344/N.

Foreign body—cerumen, food, and other debris commonly are found in the external auditory meatus.

Inflammation, chronic—frequent finding as a result of wounds or trauma (*see* Skin for additional lesions).

Inflammation, granulomatous (granulomas)—occasionally present in wall of auditory canal.

Tumors (same as skin)—occasionally observed

Neural crest tumors—resemble neurofibromas, neurofibrosarcomas, schwannomas, fibromas, and fibrosarcomas (Fischer 344/N: 1936 males 0.1%, 1983 females 0.1%)

Nerve sheath tumor, benign (schwannoma) (Sprague-Dawley: 2 reported in 585 males 0.3%)

Nerve sheath tumor, malignant (schwannoma) (Sprague-Dawley: 1 reported in 585 females 0.2%)

Middle ear—inflammation (otitis media) is occasional finding, often associated with sinus and upper respiratory tract infections; tympanic cavity usually contains purulent exudate.

Inner ear—inflammation (otitis interna) extending from middle ear; can result in encephalitis and loss of balance or circling behavior.

Zymbal's gland (auditory sebaceous glands)—holocrine glands at the base of the ear.

Tumors—commonly observed in aged rats; have squamous, sebaceous, or mixed patterns.

Adenoma, sebaceous—very low incidence

(Fischer 344/N: 2 reported in 1949 males 0.1%)

(Sprague-Dawley: 1 reported in 585 males 0.2%)

Adenocarcinoma, sebaceous—very low incidence (Sprague-Dawley: 1 of 585 males 0.2%, 1 of 585 females 0.2%)

Carcinoma, sebaceous—low incidence (Fischer 344/N: 1936 males 1.4%; 1983 females 0.7%)

Squamous cell papilloma (Fischer 344/N: 2 reported in 1936 males 0.1%)

Epididymis

Changes commonly observed are secondary to testicular atrophy and include lack or diminished spermatozoal contents, intraepithelial cyst formation, and tubular debris.

Inflammation, granulomatous (spermatic granuloma)—common.

Tumors—very low incidence

Hemangioma (angioma, hemangioendothelioma) (Fischer 344/N: 1 reported in 1936 males 0.1%)

Sarcoma (Fischer 344/N: 2 reported in 1949 males 0.1%)

Esophagus

Impaction—sporadic finding of distention with food and debris in some colonies; seldom seen in Sprague-Dawley.

Inflammation—common in association with gavage injuries during dosing; severity and frequency range from small foci of macrophages to large areas of necrosis and abscess formation; perforations can lead to extensive inflammation of subcutis or pleura; myositis, usual occurrence 2%–6%.

Tumors—very low incidence: squamous cell carcinomas have been reported (Fischer 344/N: 1 reported in 1850 males 0.1%, 1 reported in 1836 females 0.1%).

Eye

Cataract—common lens finding in aged rats.

Chromodacryorrhea—excessive red secretions of apparently normal lacrimal and Harderian glands, which may be mistaken for blood, and increased secretions are associated with stress.

Coloboma—a congenital defect due to persistence of choroid tissue with protrusion of the retina into the optic nerve sheath.

Corneal dystrophy (hyalinization or mineralization)—low-frequency findings in rats that are poorly defined, including hyalinization or mineralization (calcification) in Bowman's membrane or the anterior corneal stromal subepithelial mineralization in diabetic rats and thickening of Descemet's membrane.

Inflammation, conjunctiva (conjunctivitis)—mild nonspecific inflammation is common; severe inflammation is most commonly associated with infections caused by the sialodacryoadenitis virus.

Inflammation, cornea (keratitis)—usually is associated with inflammation of the conjunctiva; minimal infiltrates by inflammatory cells and edema can result from mild abrasions, slight trauma, or drying (epithelial desiccation).

Inflammation, periorbital (periorbititis)—usually is associated with trauma during blood sampling of the orbital sinus.

Retinal atrophy—frequent finding from a variety of causes including age, strain, ambient light exposure, temperature, nutrition, and retinal inflammation with glaucoma. The retina of the albino rat is particularly sensitive to the effects of high light intensity that results in bilateral atrophy.

Retinal folds or rosettes—infolding of all retinal layers is occasionally seen, presumably having congenital origin or associated with acquired retinal detachment.

Synechia—adhesion or attachment of the iris margin to the anterior cortex of the lens or the corneal endothelium; may occur as a congenital condition or follow inflammation in the anterior chamber.

Tumors—intraocular tumors are very rare in rats:

Glioma (Fischer 344/N: 1 reported in 1949 males 0.1%).

Leiomyosarcoma (Sprague-Dawley: 1 reported in 585 males 0.2%).

Squamous cell carcinoma of the conjunctiva is occasionally seen in aged rats (Fischer 344/N: 1 reported in 1936 males 0.1%).

Forestomach (stomach, nonglandular)

Erosion (*see* Ulcer and Necrosis).

Diverticulum—rare finding.

Dysplasia—often associated with hyperplasia.

Ulcer—erosions and ulcers (focal necrosis) with accompanying inflammation of the squamous mucosa are

very common lesions; vary in severity; severe ulceration is accompanied by acute and chronic inflammation, penetration of the inflammatory changes into the stomach wall, and, in some animals, peritonitis.

Hyperkeratosis—associated with hyperplasia.

Hyperplasia, squamous (acanthosis, hyperkeratosis, parakeratosis)—most proliferative or hyperplastic changes of the squamous mucosa are reactive, focal, and associated with gastric inflammation; occasionally the entire forestomach is affected; focal hyperplasia commonly involves the junction with the glandular stomach; may have extensive dysplasia.

Inflammation (gastritis)—common finding often associated with erosion or ulceration; varies from focal acute or chronic inflammation to perforation of the stomach wall.

Necrosis—superficial necrosis (erosion) and deep necrosis (ulceration) are common findings (*see* Ulcer).

Tumors—squamous cell tumors are uncommon:

Squamous papillomas—low incidence (Sprague–Dawley: 583 males 0.2%, 585 females 0.3%) (Fischer 344/N: 1912 males 0.2%, 1955 females 0.3%); incidence after gavage with corn oil vehicle (Fischer 344/N: 1924 males 0.3%, 1936 females 0.4%)
Squamous carcinomas—very low incidence (Fischer 344/N: 2 reported in 1912 males 0.1%, 1 reported in 1955 females 0.1%)

Leiomyosarcoma—very low incidence (Fischer 344/N: 1 reported in 1912 males 0.1%)

Harderian gland—well developed in rats

Chromodacryorrhea—excessive red secretions of apparently normal Harderian glands that may be mistaken for blood; increases associated with stress.

Atrophy, focal or diffuse (glandular ectasia)—common finding; consists of cystic dilatation of glandular lumens.

Cysts—occasional finding.

Hyperplasia, focal—occasional finding of small, non-compressive lesions with both cellular hyperplasia and hypertrophy.

Inflammation—common finding; focal glandular atrophy, fibrosis, and chronic inflammatory cells or necrosis can be a result of orbital bleeding; severe inflammation with necrosis and squamous metaplasia is most commonly associated with infections caused by the sialodacryoadenitis virus.

Tumors—uncommon but occasionally seen in aged rats

Adenoma—very low incidence
(Fischer 344/N: 1 reported in 1936 males 0.1%, 2 reported in 1950 females 0.1%)
(Sprague–Dawley: 1 reported in 390 males <0.1%)

Adenocarcinoma—very rare; none reported in the Fischer 344/N or Sprague–Dawley rat studies

Heart

Atrial thrombosis—sporadic finding in the right atrium (Sprague–Dawley: 2000 males <1%, 2000 females <1%).

Degeneration, myocardial—occasional finding in aged rats associated with necrosis or metaplasia.

Degeneration, valvular—frequent finding in aged rats characterized by myxomatous or basophilic changes.

Fibrosis—usually associated with necrosis (Sprague–Dawley: 2000 males 15.0%, 2000 females 5.8%).

Fibrosis, subendocardial—proliferation of mesenchymal cells, fibroblasts, in the subendocardium primarily involving the left ventricle that increases with age.

Hypertrophy, myocardial—usually a diffuse change that may require weights of the total heart and the individual ventricles as well as measurement of the thicknesses of the chamber walls for characterization; focal hypertrophy can be observed in areas of inflammation and fibrosis.

Inflammation (myocarditis)—usually incidental focal small aggregates of inflammatory cells and fibrosis or in association with necrosis and infectious diseases; usual occurrence in young rats 2%–7% (Sprague–Dawley: 2000 males 13.8%, 2000 females 9.5%).

Inflammation, valvular (endocarditis)—sporadic finding often found associated with suppurative inflammation elsewhere in the body, such as the feet (pododermatitis).

Necrosis, myocardial (focal necrosis; acute, necrotizing inflammation; acute focal inflammation; focal subacute or chronic inflammation; acute focal inflammation; focal subacute or chronic inflammation; focal fibrosis)—a frequent localized lesion accompanied by inflammatory cells and fibrosis occurs in aged rats; usually present in the inner region or subendocardial zone and papillary muscles of the left ventricle, possibly related to local ischemia; also can result from bacterial infections.

Metaplasia, cartilaginous and osseous—common finding in aged rats occurring in areas of myocardial degeneration.

Pigmentation—pigment-laden macrophages and mononuclear cells with fibrosis indicate old myocardial injury.

Tumors—spontaneous primary cardiac neoplasms are rare; mesenchymal, spindle-cell tumors are most common:

Hemangioma (angioma, hemangioendothelioma)—very rare (Fischer 344/N: none in 1932 males and 1972 females)

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—very low incidence (Fischer 344/N: 2 reported in 1932 males 0.1%, 1 reported in 1972 females 0.1%)

Anitschkow cell sarcoma—very rare (Fischer 344/N: none in 1932 males and 1972 females)

Atriocaval mesothelioma—very rare (Fischer 344/N: 1 reported in 1972 females 0.1%)

Fibrosarcoma—very rare (Fischer 344/N: 1 reported in 1947 males 0.1%)

Malignant schwannoma—very low incidence (Fischer 344/N: 1932 males 0.1%, 1972 females 0.2%)

Neurofibrosarcoma—very rare (Fischer 344/N: 1 reported in 1972 females 0.1%)

Rhabdomyosarcoma—very rare (Fischer 344/N: none in 1932 males and 1972 females)

Sarcoma, unspecified—very rare (Fischer 344/N: 1 reported in 1932 males 0.1%)

Intestines, unspecified (NOS)

Arteritis—occasional finding in mesenteric and pancreatic arteries usually associated with polyarteritis.

Atrophy—loss, shortening, stunting, or atrophy of the small intestine villi results from several causes including hormonal factors, reduced food intake, or starvation; focal atrophy of the large intestine mucosa occurs occasionally in colon without other apparent lesions.

Congestion—mucosal blood vessels become congested secondary to inflammation and occur as agonal changes.

Dilatation—frequent finding in animals found dead, most commonly from bacterial gas production.

Dilatation of the jejunum, ileum, or cecum—associated with paralytic ileus; can be a delayed response to intra-peritoneal administration of an anesthetic agent such as chloral hydrate; must be differentiated from Tyzzer's disease megaloileitis.

Diverticulum or pouch of small intestine—persistence of fetal yolk sac attachment in Sprague–Dawley; herniation along the mesenteric attachment in Fischer 344 rat.

Ectopic pancreatic tissue—sporadic finding in the duodenum and jejunum.

Fibrosis—occasional finding in the colon mucosa.

Hyperplasia and hypertrophy—focal reactive hyperplasia or hypertrophy of the intestinal epithelium occurs around areas of inflammation, ulceration, and heavy parasitic infestation; mucosal hypertrophy of both the large and small intestine occurs under some physiological conditions such as lactation (Sprague–Dawley: 2000 males 1.5%, 2000 females 1.6%).

Hyperplasia, lymphoid—occasional finding of non-neoplastic proliferation of cells, predominantly lymphocytes, resulting from inflammation and other immunological stimulation; normal lymphoid follicles have prominent germinal centers in the gastrointestinal tract of rats; therefore, hyperplasia should be reserved for significant increases.

Inflammation, large (colitis)—nonspecific inflammation is common; unless definite evidence of active inflammation is present, a slight increase in inflammatory cells in the lamina propria would be considered within normal limits; acute inflammation, ulceration, and necrosis (necrotizing inflammation) can occur in Tyzzer's disease, salmonellosis, and other bacterial infections.

Inflammation, small (duodenitis, jejunitis, ileitis)—uncommon finding in most laboratory colonies; can be a result of several bacterial and parasitic infections as well as nonspecific stress; vary in type, severity, and duration including erosion, ulceration, necrosis, acute and chronic active inflammation, fibrosis, reactive lymphoid

hyperplasia, reactive epithelial hyperplasia, lymph node inflammation, and peritonitis.

Mineralization (calcification)—foci associated with inflammation and necrosis occur sporadically in the muscularis externa.

Metazoan parasites

Protozoa—*Giardia muris*, *Hexamitis muris*, and *Trichomonas* species occur in the small intestine usually with no apparent effect.

Nematodiasis—pinworms (*Syphacia muris*) are common findings in the cecum and colon of rats with no inflammation; usual occurrence in young rats 3%–8%.

Tumors, large intestines, unspecified (NOS)

Adenoma (adenomatous polyp, villous adenoma)—very low incidence

(Fischer 344/N: 2 reported in 1949 males 0.1%)

(Sprague–Dawley: 1 reported in 576 females 0.2%)

Adenocarcinoma—very low incidence

(Fischer 344/N: 2 reported in 1936 males 0.1%)

(Sprague–Dawley: 1 reported in 569 males 0.2%)

Fibroma—very low incidence (Fischer 344/N: 1 reported in 1936 males 0.1%)

Fibrosarcoma—uncommon finding (Fischer 344/N: 1 reported in 1949 males 0.1%)

Lipoma—very low incidence (Fischer 344/N: 1 reported in 1950 females 0.1%)

Malignant schwannoma—very low incidence (Fischer 344/N: 1 reported in 1936 males 0.1%)

Rhabdomyosarcoma (anus)—(Sprague–Dawley: 1 reported in 585 males 0.2%)

Tumors, small intestines, unspecified (NOS)

Adenoma (adenomatous polyp)—very low incidence (Fischer 344/N: 1 reported in 1887 males 0.1%)

Adenocarcinoma—very low incidence

(Fischer 344/N: 7 reported in 1865 males 0.4%)

(Sprague–Dawley: 1 reported in 556 females 0.2%)

Fibrosarcoma—uncommon finding (Fischer 344/N: 1 reported in 1865 males 0.1%, 1 reported in 1939 females 0.2%)

Hemangioma—very rare (Fischer 344/N: one reported in a female rat < 0.1%)

Hemangiosarcoma—uncommon finding (Fischer 344/N: 1 reported in 1353 males 0.7%)

Leiomyoma—very low incidence (Fischer 344/N: 1 reported in 1865 males 0.1%)

Leiomyosarcoma—uncommon finding

(Fischer 344/N: 3 reported in 1865 males 0.2%, 2 reported in 1939 females 0.1%)

(Sprague–Dawley: 556 males 0.4%, 556 females 0.4%)

Malignant lymphoma—occasionally seem to arise from gastrointestinal lymphoid tissue (Peyer's patches)

Malignant schwannoma—very low incidence (Fischer 344/N: 1 reported in 1914 females 0.1%)

Sarcoma, unspecified—uncommon finding

(Fischer 344/N: 1 reported in 1865 males 0.1%, 1 reported in 1939 females 0.1%)

(Sprague–Dawley: 1 reported in 556 females 0.2%)

Islets, pancreatic (islets of Langerhans; pancreas, endocrine)
Atrophy, beta cell—diabetes mellitus is generally very rare in rats; genetically spontaneous occurrence in some strains, for example, BB rat.

Inflammation—spontaneous inflammation of the pancreas usually spares the islets; occasional mononuclear cells, focal chronic inflammation, can be observed in the islets of aged rats.

Hyperplasia—commonly aging rats develop large islets; size seems to be influenced by *ad libitum* feeding, obesity, and repeated breeding; characterized by several multilobular or multinodular large and pleomorphic islets (Sprague–Dawley: 583 males 5.5%, 585 females 3.9%).

Tumors

Islet cell adenoma—low incidence

(Fischer 344/N: 1868 males 3.2%, 1934 females 1.0%)

(Sprague–Dawley: 583 males 7.5%, 585 females 3.9%)

Islet cell carcinoma—low incidence

(Fischer 344/N: 1868 males 2.1%, 1934 females 0.3%)

(Sprague–Dawley: 583 males 1.9%, 585 females 1.0%)

Joints

Ankylosis—fusion of the joint with immobility resulting from chronic inflammation

Degeneration, mucinous or chondromucinous, cystic—a common lesion of the cartilage, especially in the sternum, but also other sites involving articular and growth plate cartilage

Inflammation (arthritis)—sporadic lesions, usually adjacent to wounds or septic foci

Osteoarthritis (osteoarthrosis, degenerative joint disease)—a complex of lesions characterized by erosion of the articular cartilage, degeneration, and inflammation

Tumors

Synovial sarcoma (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1950 females 0.1%)

Kidney

Bowman's capsule changes (*see* Metaplasia).

Cysts—common finding associated with dilatation of tubules that may occur individually, but usually accompany renal nephritis and nephrosis.

End-stage—marked or severe glomerular, tubular, and interstitial changes with inflammation.

Extramedullary hematopoiesis—sporadically observed in adult rats at times of increased demand for bone marrow activity.

Glomerulonephritis and glomerulosclerosis—usually associated with nephropathy (*see* Nephropathy).

Hydronephrosis (pelvis dilatation)—common finding; has both acquired and hereditary causes; usual occurrence in young rats 10%.

Hyperplasia, pelvis—focal thickening of the urinary epithelium accompanies mineralization and pelvic inflammation.

Hyperplasia, tubular epithelium, focal—occasional finding (Sprague–Dawley: 585 males 0.2%, none in 584 females).

Inclusions, tubular (protein absorption or hyaline droplets)—hyaline droplets, PAS positive, occur occasionally in the cytoplasm of renal tubular cells of apparently normal kidneys from males; usual occurrence in young males 15%.

Infarction—occasional finding resulting from metastatic tumors, septic thrombosis, or advanced mononuclear cell leukemia.

Inflammation (nephritis, pyelonephritis)—a variety of inflammatory changes involve various portions of the kidney of rats; the most important inflammatory findings are associated with nephropathy (*see* Nephropathy); changes of the pelvis are often associated with mineralization, hydronephrosis, and pyelonephritis; acute pyelonephritis is usually associated with ascending lower urinary tract infections and uncommon in most research laboratories; embolic suppurative nephritis is infrequent.

Inflammation, chronic—common finding of small lymphoid aggregates in interstitial tissues and in the renal pelvis beneath the transitional epithelium; usual occurrence in young rats, males 12%, and females 7%.

Metaplasia, Bowman's capsule—lining of the capsule by high cuboidal epithelium resembling the proximal tubule; also termed hyperplasia and adenomatoid transformation.

Mineralization (calcification, calcium deposits, lithiasis, nephrocalcinosis)—mineral deposits are common in the urinary bladder, renal pelvis, and renal tubules; mineral deposits may be seen in the renal papilla and pelvic mucous membrane that are often accompanied by focal fibrosis, granuloma formation, and ulceration or hyperplasia of the overlying transitional epithelium; intratubular deposits consist generally of small concretions in the lumens that are found commonly in females at the corticomedullary junction of otherwise-normal kidneys; usual occurrence in young female rats 6% (Sprague–Dawley: 2000 males 3.5%; 2000 females 27%); mineralization of large arteries is the most common vascular lesion; associated with nephropathy.

Nephropathy (chronic nephrosis, chronic nephritis, chronic progressive nephrosis, glomerulonephritis, glomerulonephrosis, glomerulonephropathy, glomerulosclerosis, glomerular hyalinosis)—very common and important renal disease in adult and aged rats; can be observed as early as 5 months; more frequent and severe in males than females; can become a problem in studies that exceed 2 years' duration; strain differences have been observed; OFA-ICO rats more affected than Sprague–Dawley.

(Sprague–Dawley: 2000 males 56%, 2000 females 23.2%)

(Long–Evans: males 74%, females 44%)

Pigmentation, tubular—iron-positive brownish pigment granules are commonly observed in the tubular epithelial cells of kidneys from rats older than 1 year of age.

Pyelonephritis—sporadic finding usually associated with inflammation of the lower urinary tract; more frequent in males accompanying inflammation of the prostate, seminal vesicles, coagulation gland, and urinary bladder.

Regeneration, tubular—a common finding, predominantly in males; often an early stage of progressive nephropathy; usual occurrence in young rats, males 32%, females 4% (Sprague–Dawley: 2000 males 15.5%; 2000 females 14.9%).

Tumors

Leiomyosarcoma (Fischer 344/N: 1 reported in 1943 males 0.1%)

Lipoma (lipomatous tumors, lipomatous hamartoma, angioliipoma, myoliipoma, angiomyoliipoma, hamartoma, mixed tumors)—very low incidence (Fischer 344/N: 2 reported in 1928 males 0.1%, 1 reported in 1944 females 0.1%) (Sprague–Dawley: 585 males 0.5%, 584 females 0.3%)

Liposarcoma (malignant mixed tumors) (Fischer 344/N: 1 reported in 1943 males 0.1%) (Sprague–Dawley: 585 males 0.7%, none in 584 females)

Nephroblastoma—very low incidence (Fischer 344/N: 1 reported in 1928 males 0.1%) (Sprague–Dawley: none in 585 males, 584 females 0.2%)

Polyp (Sprague–Dawley: 1 reported in 585 males 0.2%)

Sarcoma, unspecified (Fischer 344/N: 1 reported in 1928 males 0.1%)

Tubular cell adenoma—very low incidence (Fischer 344/N: 1928 males 0.4%, 1977 females 0.1%) (Sprague–Dawley: 585 males 0.2%, none in 584 females)

Tubular cell adenocarcinoma—very low incidence (Fischer 344/N: 3 reported in 1928 males 0.2%, 2 reported in 1977 females 0.1%) (Sprague–Dawley: 585 males 0.2%, none in 584 females)

Transitional cell papilloma—very low incidence (Fischer 344/N: 3 reported in 1928 males 0.2%)

Transitional cell carcinoma—very low incidence (Fischer 344/N: 2 reported in 1928 males 0.2%)

Lacrimal gland (*see* Harderian Gland)

Tumor

Adenoma—very low incidence (Fischer 344/N: 1 reported in 1983 females 0.1%)

Larynx (also *see* Trachea)

Calcification (mineralization), ossification—the laryngeal cartilage may become focally calcified or ossified at any age in the rat.

Inflammation, acute or chronic (laryngitis)—frequent findings of small foci of inflammatory cell infiltrates; submucosal aggregates of lymphoid cells are common in untreated rats; may be involved as a part of a variety of either upper or lower respiratory tract infections.

Metaplasia, squamous—the laryngeal mucosa of the rat is particularly sensitive to inhaled irritants and responds by undergoing squamous metaplasia.

Liver

Angiectasis (peliosis hepatis, telangiectasis)—common finding especially in male Fischer 344 rats; widely dilated vascular spaces lined by normal-appearing endothelium that results in blood-filled cystic spaces and distortion of the sinusoids (Sprague–Dawley: 2000 males 13.1%, 2000 females 15.4%).

Atrophy, hepatocyte—occasional finding resulting from local anoxia during angiectasis or centrilobular congestion in mononuclear cell leukemia and malnutrition.

Congestion—dilatation of sinusoids is a common finding dependent on the mode of death and length of time before tissue fixation; frequently centrilobular and more pronounced after cardiac failure; simple periportal sinusoidal is characterized by dilated spaces conforming in position and shape to normal-appearing sinusoids.

Cirrhosis—unusual finding of regenerating nodules of hepatocytes within encircling bands of fibrous tissue and associated cell necrosis.

Cytomegaly—common finding in hepatocytes of aged rats.

Degeneration—occurs in a variety of forms associated with metabolic changes, necrosis, and inflammation.

Degeneration, cystic, focal (spongiosis hepatis, cystic focus, focal cystic change)—common finding in aged rats in which multilocular cystic lesions contain a finely granular or flocculent eosinophilic material; the cyst-like cavities are not lined by epithelial or endothelial cells and occasionally contain erythrocytes.

Dilatation, sinusoidal (*see* Angiectasis and Congestion)

Enlargement (*see* Hepatocytic hypertrophy)

Extramedullary hematopoiesis—frequent finding as foci along sinusoids or around central veins and portal vessels.

Clear cell change—diffuse clear cell change may be seen in nonfasting normal, well-fed rats associated with storage of glycogen; focal clear cell changes are a form of the cellular alterations.

Fatty change (fatty metamorphosis, lipidosis)—fat vacuoles in individual hepatocytes occur spontaneously in random patterns and increase with age; in foci of cellular alteration, fatty changes are termed vacuolated; diffuse fatty changes occur occasionally in aged or sick animals or associated with severe or metastatic neoplasia (Sprague–Dawley: 2000 males 23.5%, 2000 females 16.9%).

Fibrosis—common finding associated with cirrhosis and a variety of degenerative, necrotic, and inflammatory lesions.

Fibrosis, peribiliary—common in aging rats and often involves only a few portal triads.

Hepatocytic cellular alteration, focal—frequent finding of foci from one to several lobules in diameter consisting of hepatocytes; identified as clear cell, eosinophilic, basophilic, vacuolated, and mixed cell foci (Sprague–Dawley: 585 males 21.2%, 585 females 22.2%) (Sprague–Dawley: 2000 males 23.4%, 2000 females 22.4%).

Hepatodiaphragmatic nodule (HDN)—common developmental anomaly in Fischer (F344) strain (1%–11%), rare in other strains; usually a rounded mass protruding from diaphragmatic surface of median lobe.

Hyperplasia, hepatocellular (hyperplastic nodule)—occasional finding of focal areas from a few cells to several larger than a lobule consisting of normal or hypertrophic hepatocytes, portal tracts, and bile ducts (Sprague–Dawley: 2000 males 26.2%, 2000 females 28.1%).

Hypertrophy, hepatocytic—the most common form is cytomegaly in aged rats; less common finding in aged rats associated with degeneration, necrosis, or inflammation.

Inclusion bodies, intranuclear—uncommon finding in hepatocytes of aged rats resulting from invaginations of the cytoplasm.

Inflammation (hepatitis)—very common incidental finding (Sprague–Dawley: 2000 males 6.2%, 2000 females 4.2%); up to 80% of young rats have small aggregates of lymphoid cells or chronic inflammation and granulomas scattered in the liver; larger foci are associated with coagulative necrosis that rarely may be related to *B. piliformis* (Tyzzer's disease) or salmonellosis in laboratory rats; chronic inflammation must be distinguished from extramedullary hematopoiesis, leukemias, and lymphomas.

Karyomegaly—common finding in hepatocytes of aged rats.

Necrosis—sporadic finding usually associated with inflammation, neoplasia, or systemic disease (Sprague–Dawley: 2000 males 6.1%, 2000 females 5.4%); individual cell necrosis (apoptosis) of single or small aggregates of hepatocytes is a sporadic finding; massive necrosis (infarction) is usually a result of vascular occlusion.

Parasites—*Cysticercus fasciolaris* is the larval stage of *Taenia taeniaeformis*, forms cystic cavities with dense fibrous walls attached to the liver, and can result in the formation of sarcoma.

Peliosis hepatis (*see* Angiectasis)

Pigmentation—a variety of pigments occur in the liver; lipofuscin (ceroid) in hepatocytes, bile pigment in canaliculi and ducts, and hemosiderin in Kupffer cells.

Spongiosis hepatis (*see* Degeneration, cystic)

Tumors—primary tumors have low frequency in rats:

Hemangioma (angioma, hemangioendothelioma)—Fischer 344/N: 1 reported in 3569 males <0.1%.

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—occasional (Sprague–Dawley: none in 585 males, 585 females 0.3%).

Hepatoblastoma—rare finding, may be undifferentiated variant of hepatocellular carcinoma; often concurrent with other hepatocellular tumors.

Hepatocellular adenoma (including neoplastic nodule)—low incidence

(Fischer 344/N: 1928 males 4.1%, 1979 females 2.3%)

(Sprague–Dawley: 585 males 4.6%, 585 females 5.8%)

Hepatocellular carcinoma—uncommon in Sprague–Dawley and Fischer 344 strains

(Fischer 344/N: 1928 males 1.0%, 1979 females 0.2%)

(Sprague–Dawley: 585 males 2.4%, 585 females 0.2%)

Histiocytic sarcoma (malignant histiocytoma)—low incidence; often involves the liver and sometimes the liver appears to be the primary site.

Histiocytoma, fibrous (Fischer 344/N: 1 reported in 1350 males 0.07%, 1 reported in 1351 females 0.07%).

Lipoma—very low incidence (Fischer 344/N: 1 reported in 1928 males 0.1%).

Neoplastic nodule—a term used by some pathologists that combined the benign hepatocellular proliferative lesions, hyperplasia, and adenoma (*see* Hepatocellular adenoma).

Sarcomas—most nonvascular sarcomas are rare in the liver; the majority reported have been associated with the larval tapeworm, *C. fasciolaris*.

Secondary tumors—a large variety of malignant tumors may metastasize or have multicentric involvement of the liver, especially leukemia and lymphoma.

Bile ducts

Cholangiofibrosis (adenofibrosis)—characterized by excessive proliferation of fibrous tissue and bile ducts; uncommon in rats.

Cholestasis—sporadic finding usually associated with inflammation of liver and bile ducts.

Cyst—solitary and multiple cysts commonly occur in livers of aged rats.

Fibrosis, focal (peribiliary fibrosis, peribiliary sclerosis)—common in aging rats; often involves only a few portal triads.

Hyperplasia—common in old rats; may be cystic; often accompanied by peritubular fibrosis and inflammatory cells; reduced by food restriction.

Inflammation (cholangitis)—occasional finding of acute inflammatory cells increases with age; increased by bile duct obstruction and cholestasis; frequently accompanies bile duct hyperplasia and peritubular fibrosis.

Tumors—uncommon in rats

Cholangioma (bile duct adenoma)—uncommon

(Fischer 344/N: 1 reported in 1928 males 0.1%)

(Sprague–Dawley: 585 males 0.2%, 585 females 0.2%)

Cholangiocarcinoma—very low incidence

(Fischer 344/N: 1 reported in 1946 males 0.1%, 2 reported in 1945 females 0.1%)

(Sprague–Dawley: 585 males 0.2%, none in 585 females)

Cholangiosarcoma—none in 7137 Fischer 344/N or 1170 Sprague–Dawley rats

Lung

Abscess—occasional finding, usually in aged animals; results from local or systemic infections.

Atelectasis—sporadic finding of collapsed alveoli resulting from obstructive bronchial disease or neoplasms.

Bronchiectasis—dilatation of bronchi or bronchioles usually results from infections with chronic inflammation; may be accompanied by squamous metaplasia.

Bronchopneumonia—occasional finding in aged rats; frequent in younger animals on gavage experiments.

Congestion—frequent finding associated with agonal changes related to the mode of death; marked in animals found dead; accompanied by edema in cardiac failure.

Chronic venous congestion—occasional finding associated with thrombosis of the left atrium; accompanied by alveolar septa thickening and fibrosis (fibrosing alveolitis or interstitial pneumonia).

Edema—sporadic accompanying cardiac failure.

Emphysema—focal emphysema may be found associated with inflammation and fibrosis or spontaneously in certain susceptible animals.

Fibrosis—occasionally found as focal lesions in association with chronic and granulomatous inflammation; diffuse segmental fibrosis (fibrosing alveolitis) secondary to chronic venous congestion.

Hemorrhage—frequent finding associated with agonal changes related to the mode of death or method of sacrifice.

Hyperplasia, alveolar epithelium, focal (adenomatous hyperplasia, pulmonary adenomatosis, alveolar cell hyperplasia, epithelialization)—frequent finding, usually multicentric, increases with increased age.

Hyperplasia, alveolar macrophage (foam cells, histiocytosis, lipidosis)—common finding of collections of foamy alveolar macrophages in older rats that may be associated with chronic or granulomatous inflammation and cholesterol clefts, may have fine particulate pigment; usual occurrences in young rats 16%–20%.

Hyperplasia, goblet cell—hypersecretion by mucous cells of the bronchial epithelium associated with chronic inflammation.

Hyperplasia, lymphoid—small aggregates of lymphocytes occur around proximal parts of major bronchi as part of the bronchial-associated lymphoid tissue; increased lymphoid cells are common in association with inflammation and after viral and mycoplasma respiratory tract infections.

Inflammation, acute or chronic (alveolitis, bronchitis, bronchopneumonia, pneumonia)—very common to have small aggregates of lymphoid cells around bronchioles and small vessels; usual occurrence in young rats up to 56%; more extensive inflammation, alveolitis,

bronchiolitis, or pneumonitis occurs in young rats at rates of 18%–20%.

Inflammation, granulomatous (granuloma)—occasional finding resulting from infections, inhaled foreign bodies, and aggregates of alveolar (foamy) macrophages with cholesterol deposits.

Metaplasia, squamous cell—common in bronchi in association with chronic inflammation.

Pigmentation—pigment-laden macrophages usually containing iron (hemosiderin) may accumulate in the air spaces; may be associated with congestion and hemorrhage.

Tumors—uncommon in most strains of rats

Alveolar/bronchiolar adenoma—low incidence (Fischer 344/N: 1933 males 1.3%, 1974 females 0.8%) (Sprague–Dawley: 585 males 0.2%, none in 585 females)

Alveolar/bronchiolar carcinoma (adenocarcinoma)—very low incidence (Fischer 344/N: 1933 males 1.0%, 1974 females 0.3%) (Sprague–Dawley: 585 males 0.2%, none in 585 females)

Squamous cell carcinoma—very low incidence (Fischer 344/N: 1933 males 0.2%, 1945 females 0.1%).

Hemangioma (angioma, hemangioendothelioma)—rare (Fischer 344/N: 1 reported in 3567 females <0.1%)

Hemangiosarcoma (angiosarcoma, hemangioendothelioma)—Fischer 344/N: 1 reported in 3566 males <0.1%

Malignant lymphoma—sometimes the lung is the primary site.

Metastatic tumors—common site for metastatic malignant tumors.

Lymph node (unspecified, NOS)—mandibular and mesenteric are most frequently examined.

Atrophy (hypoplasia)—depletion of lymphoid cells is common in aged rats.

Congestion—common especially in mesenteric lymph nodes; can be related to the mode of death; usually agonal origin.

Extramedullary hematopoiesis (myeloid metaplasia, granulocytic metaplasia)—observed during increased hematopoietic demand; predominantly myelocytic.

Fibrosis—associated with healing of inflammation.

Granuloma (*see* Inflammation)

Hemorrhage—frequent finding; can be related to the mode of death; usually agonal in origin.

Hyperplasia—occasional finding of nonneoplastic proliferation of mixed cell types usually associated with active inflammatory lesions elsewhere in the body; consists of plasma cells, lymphocytes, macrophages, and other inflammatory cell types; usual occurrence in young rats of 26%–40%.

Hyperplasia, lymphoid (follicular hyperplasia)—a common focal nonneoplastic proliferation of cells,

predominantly paracortical lymphocytes accompanied by sinusoidal macrophages; hyperplasia of the germinal centers is a common reactive response to inflammation and systemic infections (Sprague–Dawley: 4000 males and females 13%).

Hyperplasia, plasma cell—commonly observed in sinusoids accompanying lymphoid hyperplasia.

Hyperplasia, reactive (*see* Hyperplasia, lymphoid).

Inflammation (adenitis)—occasional finding, both as primary sites and secondary sites (Sprague–Dawley: 4000 males and females 2.7%); cervical lymph nodes become involved in sialodacryoadenitis; mesenteric lymph nodes may develop acute, necrotizing, or granulomatous inflammation from *Salmonella* sp. infections.

Inflammation, granulomatous (granuloma)—small granulomas are common findings resulting from several causes including foreign materials and infections, but most often are aggregates of pigment or ceroid-laden macrophages.

Pigmentation—pigments are common finding in histiocytes or macrophages; both ceroid and iron-positive pigments may be observed.

Sinus histiocytosis—very common findings in which distended sinuses contain numerous histiocytes and free macrophages; leukocytes, lymphocytes, and plasma cells also may be present; macrophages filled with ceroid or lipochrome may be seen, particularly in the mesenteric lymph nodes.

Telangiectasis—occasional finding of prominent dilated blood vessels (*see* Congestion).

Tumors—lymphoreticular neoplasms have a high spontaneous incidence in rats:

Hemangioma (angioma, hemangioendothelioma)—benign vascular lesions are occasionally observed; some are believed to be congenital malformations and not true neoplasms; true tumors are uncommon (Sprague–Dawley: 2 reported in 581 males 0.3%).

Hemangiosarcoma (angiosarcoma, hemangioendothelioma) (Sprague–Dawley: 3 reported in 581 males 0.5%).

Histiocytic sarcoma (malignant histiocytoma)—frequently involves the liver, lymph nodes, lung, spleen, mediastinum, retroperitoneum, and subcutis; sometimes infiltrates in lymphomatous pattern; most frequent in Sprague–Dawley, Osborne–Mendel, and Wistar strains; all sites and multiple organ involvement incidence

(Fischer 344/N: 4 reported in 1353 males 0.3%)

(Sprague–Dawley: 585 males 2.6%, 585 females 1.2%)

Lymphangiosarcoma

(Sprague–Dawley: 1 reported in 581 males 0.2%)

(Sprague–Dawley: 4000 males and females 0.5%)

Leukemia—frequently occurs in some strains; occasionally observed at less than 6 months of age.

Leukemia, granulocytic (myeloid leukemia)—relatively common in aged rats, can occur spontaneously in

rats less than 6 months of age; white cell counts in peripheral blood are often very high; splenic involvement may result in very large sizes; myelocytic type with neutrophils is most common; eosinophilic type is sometimes observed; granulocytic leukemia must be differentiated from leukemoid reactions associated with infections and large tumors; all sites and multiple organ involvement incidence

(Fischer 344/N: 1 reported in 1353 males 0.7%)

(Sprague–Dawley: 585 males 0.3%, 585 females 0.2%)

(Sprague–Dawley: 4000 males and females 0.7%).

Leukemia, mononuclear cell (large granular lymphocytic leukemia, monocytic leukemia, lymphosarcoma)—appears to arise in the spleen and secondarily involves the liver, bone marrow, lymph nodes, adrenal gland, and kidneys; very common in Fischer 344 rats (Fischer 344/N: 1936 males 33.6%, 1983 females 20.2%); incidence is reportedly decreased in males by gavage with corn oil vehicle (Fischer 344/N: 1949 males 17.1%, 1950 females 19.3%); mononuclear cell leukemia somewhat different from that of Fischer 344 rats has been reported with diffuse infiltration of spleen, liver, and lung in Wistar-Furth, Wistar, and other strains.

Leukemia, myeloblastic—less common form.

Leukemia, lymphocytic—high absolute lymphocyte counts in the peripheral blood resulting from increased numbers of small lymphocytes; may have extensive visceral infiltration.

Leukemia, lymphoblastic—large numbers of lymphoblasts in the peripheral blood.

Leukemia, stem cell—reported in young Sprague–Dawley rats; primarily affects the bone marrow with secondary involvement of the CNS.

Lymphoma, malignant—a variety of cell types occur in rats; both as monomorphic and mixed forms.

Lymphoma, lymphocytic (lymphosarcoma, lymphoplasmacytic lymphoma, small cell lymphoma)—lymphoma composed of well- and intermediately differentiated lymphocytes.

Lymphoma, lymphoblastic (lymphoblastic lymphosarcoma)—lymphoma composed of poorly differentiated lymphocytes.

Lymphoma, large cell (reticulum cell sarcoma, immunoblastic lymphoma)—these lymphomas occur in lymph nodes, spleen, liver, and bone marrow; rarely occurs in skin; sometimes arises as single masses (lymphosarcoma) in lung, lymph nodes of the abdominal cavity (mesenteric and ileocecal), and thymus.

Lymphoma, malignant—all types, multiple organ involvement incidence

(Fischer 344/N: 1353 males 0.37%, 1351 females 0.22%)

(Sprague–Dawley: 585 males 1.2%, 585 females 0.7%)

Plasma cell sarcoma (plasmacytoma, plasma cell lymphoma)—very low incidence finding in Sprague–Dawley rats; usually considered a form of lymphoma.

Metastatic tumors—occasional; particularly malignant epithelial tumors in adjacent or lymphatic drainage areas.

Mammary gland

Cystic change (cystic degeneration, duct ectasia)—frequent finding of cystic dilatation of ducts or ductules in the absence of hyperplasia in aged rats.

Fibrosis—usually associated with fibroadenomas; occurs occasionally as small foci surrounding otherwise-normal ducts and acini.

Galactocele—common finding; characterized by prominent cystic dilatation of ducts and large cyst-like structures; rupture of the cystic lesions results in inflammation.

Hyperplasia of duct epithelium and acini—common finding; hyperplasia of acinar epithelium (adenosis) and ductular epithelium can be pronounced; cystic hyperplasia has both epithelial hyperplasia and cystic change (Sprague–Dawley: 1 reported in 585 females 1.5%).

Inflammation (mastitis)—foci of inflammatory cells are an occasional finding; more severe inflammation including abscesses is usually associated with ruptured cysts and tumors.

Tumors

Incidence varies with strain, diet, and housing; food restriction reduces the spontaneous mammary tumor incidence; the Sprague–Dawley strain females have a high spontaneous incidence of tumors; occasionally tumors are found before 6 months of age and in males:

Adenoma—low incidence; a variety of types occur, including tubular and cystic forms (Fischer 344/N: 1936 males 0.1%, 1983 females 0.9%) (Sprague–Dawley 493 males 0.4%; 585 females 6.5%).

Adenoacanthoma—a malignant tumor consisting of both glandular and squamous cell differentiation; rare (Fischer 344/N: 1 reported in 3572 males <0.1%).

Adenocarcinoma (carcinoma)—the most common form of malignant mammary tumor in Sprague–Dawley rats; can become very large with necrosis, ulceration, and inflammation, leading to leukocytosis or leukemoid reactions; have various patterns including cribriform, tubular, papillary, solid, and in multiple combinations from well differentiated to anaplastic; sometimes squamous metaplasia is observed (adenosquamous carcinoma): (Fischer 344/N: 1936 males 0.2%, 1983 females 2.6%)

(Sprague–Dawley: 493 males 0.8%, 585 females 16.8%)

Carcinosarcoma—rare; characterized by proliferating and malignant infiltrative growth of both

glandular and spindle cell or sarcomatous elements (none in the Fischer 344/N and Sprague–Dawley studies).

Fibroadenoma—common in female rats; composed of benign fibrous and glandular tissues (Fischer 344/N: 1936 males 2.6%, 1983 females 29%) (Sprague–Dawley: 493 males 1.2%, 585 females 31.3%).

Fibroma—uncommon finding; most are fibroadenomas with atrophic or minimal glandular tissue (Fischer 344/N: 3 reported in 3572 males <0.1%, 1 reported in 3570 females <0.1%).

Fibrosarcoma—uncommon finding (Fischer 344/N: 2 reported in 1351 females 0.15%).

Intraduct papilloma—occasional finding; benign papillary growths within ducts.

Mediastinum

Ectopic thyroid tissue

Mesentery (*see also* Peritoneum)

Ectopic pancreatic tissue—sporadic finding

Fat necrosis—common finding in aged rats

Inflammation (peritonitis)—common finding of small aggregates of lymphoid cells

Tumors

Mesothelioma (Fischer 344/N: 1936 males 2.7%, 1983 females 0.1%)

Paranglioma (*see* Blood vessels) (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)

Fibroma (Fischer 344/N: 1 reported in 1936 males 0.1%, 2 reported in 1983 females 0.1%)

Fibrosarcoma (Fischer 344/N: 1 reported in 1936 males 0.1%, 2 reported in 1950 females 0.1%)

Leiomyosarcoma (Fischer 344/N: 1 reported in 1983 females 0.1%)

Lipoma (Fischer 344/N: 1 reported in 1936 males 0.1%, 2 reported in 1983 females 0.1%)

Liposarcoma (Fischer 344/N: 1 reported in 1949 males 0.1%, 1 reported in 1950 females 0.1%)

Neurofibrosarcoma (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)

Sarcoma, unspecified (Fischer 344/N: 1936 males 0.2%, 1983 females 0.2%)

Mouth (*see* Oral cavity)

Muscle (*see* Skeletal muscle)

Nose (nasal cavities, nasal sinuses)

Inflammation (rhinitis, sinusitis)—both acute and chronic inflammations are common and associated with respiratory tract infections and inhaled foreign materials; submucosal aggregates of lymphoid cells are common in untreated rats.

Tumors—very low incidence; no spontaneous nasal tumors were reported in more than 1170 Sprague–Dawley rats:

Adenoma (Fischer 344/N: 2 reported in 1949 males 0.1%)

Squamous cell papilloma (Fischer 344/N: 1 reported in 1936 males 0.1%)

Squamous cell carcinoma (Fischer 344/N: 1 reported in 1936 males 0.1%)

Squamous cell carcinoma of nasolacrimal duct (Fischer 344/N: 2 reported in 1936 males 0.1%)

Oral cavity (gingiva, nasopharynx, oropharynx, palate, pharynx)

Inflammation—nonspecific foci of inflammatory cells are occasionally observed in subepithelial tissues.

Tumors—occasional findings in oral cavity

Squamous cell papilloma (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)

Incidence after gavage with corn oil vehicle (Fischer 344/N: 1949 males 0.3%, 1950 females 0.3%)

Squamous cell carcinoma—usually highly invasive; infiltrates the surrounding structures and occasionally metastasizes to the lungs and lymph nodes (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)

Mesenchymal tumors—sporadic (Fischer 344/N: none in 1936 males and 1983 females)

Ovary

Angiectasis (angiectasia)—sporadic finding.

Atrophy—high incidence of small ovaries with increased age; wide variation in the appearance of these ovaries in aged rats; usually has decreased numbers of ova; may be accompanied with simple cysts, stromal fibrosis, interstitial cell hyperplasia, or tubular structures containing Sertoli-like cells.

Corpus luteum—normal corpora lutea can be large; must be differentiated from luteal-granulosa cell tumor or luteoma.

Cyst, all types—common with occurrence of several types.

Dilatation of ovarian bursa is normal as during the estrus cycle but may be mistaken for cysts.

Follicular and luteal—common; appear to develop in rats when estrus cycle becomes irregular with age.

Hemorrhagic or hematomas—occasional.

Parovarian (*see* Oviduct).

Simple—cysts lined by simple cuboidal or tubular epithelium; common in mature and aged females.

Hyaline degeneration—usually affects corpora lutea; may undergo mineralization.

Hyperplasia, granulosa cell—occasional focal finding.

Hyperplasia, interstitial cell—a common aging change; must be differentiated from the relative increase associated with atrophy.

Hyperplasia, sertoliform (*see* Atrophy).

Hyperplasia, epithelial cell (tubular)—uncommon in Fischer 344 rat; must be differentiated from neoplasms.

Ovotestes—rare dysgenic developmental abnormalities in which female rats possess bilateral ovarian tissue and

nonfunctional testicular tissue and epididymides have been reported in Sprague-Dawley rats.

Pigmentation, ceroid (lipofuscin)—common; hemosiderin may also be present, usually associated with vascular changes.

Tumors, ovarian tumors are observed occasionally in aged rats; classification and histogenesis are often uncertain; terminology is sometimes imprecise and varies widely.

Tumors—serosal or epithelial

Cystadenoma—very low incidence (Fischer 344/N: 1 reported in 1928 females 0.1%)

Tubular adenoma (Fischer 344/N: 1 reported in 1928 females 0.1%)

Tubular adenocarcinoma—none in 3886 Fischer 344/N strain

Adenocarcinoma and cystadenocarcinoma—rare in Fischer 344/N

Carcinoma, unspecified (NOS)—(Fischer 344/N: 2 reported in 1928 females 0.1%)

Tubulostromal adenoma and adenocarcinoma—rare

Mesothelioma—rare in Fischer 344/N rat; arise within ovarian bursa

Tumors, sex cord

Granulosa cell tumors (include thecoma and luteoma)—can vary from almost pure granulosa cell to pure theca cell tumors; predominantly benign and unilateral

Granulosa cell tumors—most common ovarian neoplasms in Fischer 344/N strain

(Fischer 344/N: in 1958 females; benign 0.7%; malignant 0.2%)

(Sprague-Dawley: 1 reported in 572 females 0.2%)

Thecoma (Fischer 344/N: 3 reported in 1928 females 0.2%)

Luteoma (Fischer 344/N: 2 reported in 1958 females 0.1%)

Sertoli/Leydig cell tumors (androblastoma, arrhenoblastoma)—rare in aged rats (Sprague-Dawley: 1 reported in 572 females 0.2%)

Sertoli cell tumor—rare (Fischer 344/N: 1 reported in 1958 females 0.1%)

Gonadal stromal tumor (undifferentiated theca-granulosa cell tumors)—low incidence (Fischer 344/N: 1 reported in 1928 females 0.1%)

Tumors, germ cell

Teratoma—rare; none in 25,000 Fischer 344/N rats; none reported in more than 1170 Sprague-Dawley rats.

Dysgerminoma—rare; none in 3886 Fischer 344/N rats; one reported in Wistar strain.

Choriocarcinoma—very rare; one reported in 25,000 Fischer 344/N rats.

Ovarian yolk sac carcinoma—very rare; a few have been reported in Fischer 344/N rats and other strains.

Tumors, mesenchymal (connective) tissue

Lipoma (Fischer 344/N: 1 reported in 1928 females 0.1%)

Fibroma (Fischer 344/N: 1 reported in 1928 females 0.1%)

Sarcoma (Fischer 344/N: 1 reported in 1958 females 0.1%)

Metastatic tumors—sporadic findings usually associated with malignant tumors of the peritoneal cavity; the most common is malignant histiocytoma (histiocytic sarcoma).

Oviducts—lesions are often associated with changes in ovary or uterus.

Cysts—common finding in mature and aged rats; must be differentiated from normal dilatation of bursa during estrous cycle; parovarian cysts around the ovaries probably arise from remnants of the paramesonephric or mesonephric ducts.

Dilatation—normal in mature rats (*see* Cysts).

Inflammation (salpingitis)—uncommon in rats, can result from mycoplasma infections.

Tumor

Leiomyoma—very rare; none in 3933 Fischer 344/N and 585 Sprague–Dawley rats.

Pancreas (pancreas, exocrine)

Arteritis (polyarteritis nodosa, periarteritis, perivasculitis)—occasional finding in aged rats (*see* Blood vessels); peritoneal inflammation and malignant neoplasms may result in thrombosis and arteritis.

Atrophy—common finding usually accompanying inflammation; diffuse atrophy can result from a variety of other causes, including starvation, malnutrition, zinc or copper deficiencies, and protein deprivation; characterized by diffuse loss of exocrine tissue with a general absence of inflammation and replacement by fatty tissue.

Cyst—cystic dilatation of the pancreatic duct occurs occasionally; often associated with dilated bile ducts or acute inflammation.

Cytoplasmic vacuolization—common finding in aged rats, apparently due to the presence of lipid droplets.

Dilated ducts—occasional finding (*see* Cyst).

Edema, interstitial—sporadic finding usually associated with acute inflammation, cardiac failure, or as an age-related change.

Eosinophilic change, focal—small irregular nonneoplastic foci of acinar cells having increased cytoplasmic eosinophilia or decreased (usual) cytoplasmic basophilia; may affect parts of single or several lobules.

Fibrosis, interstitial—usually associated with chronic inflammation (Sprague–Dawley: 2000 males 6.4%, 2000 females <1%).

Hyperplasia, acinar, focal (hyperplastic nodules)—sporadic finding in untreated rats; usually small numbers and multiple (Sprague–Dawley: 583 males 0.3%, none in 585 females).

Hyperplasia, focal of pancreatic duct—occasional finding.

Inflammation (pancreatitis)—Sprague–Dawley: 2000 males 3.8%, 2000 females 2.8%; acute inflammation occurs spontaneously as a sporadic finding in rats with leukocytes in the ducts and ductules, focal acinar cell inflammation, congestion, and interstitial edema; the duct epithelium can become very hyperplastic; fat necrosis may occur; chronic inflammation (chronic relapsing pancreatitis) usually occurs associated with lobular atrophy; early focal mononuclear intralobular infiltrates; later acinar cell degeneration and necrosis may lead to cystic (microcystic changes) dilatation of ducts and ductules (ectasia); fibrosis of interstitium; increased numbers of fat cells may replace one or more lobules; incidence appears to be influenced by strain; infrequent in Sprague–Dawley, 40% in Long–Evans strain.

Pigmentation—occasional finding, usually iron-positive, associated chronic inflammation; hemosiderin may occur around islets.

Tumors—occasional in aged rats

Acinar cell adenoma—very low incidence (Sprague–Dawley: 583 males 0.7%, none in 585 females)

(Fischer 344/N: 1868 males 0.3%, 1934 females 0.2%) incidence was reportedly increased in males by gavage with corn oil vehicle

(Fischer 344/N: 1865 males 5.4%, 1875 females 0.4%)

Acinar cell carcinoma (adenocarcinoma)—rare (Fischer 344/N: none in 1868 males, none in 1934 females); incidence was reportedly increased in males by gavage with corn oil vehicle

(Fischer 344/N: 1865 males 0.3%, none in 1875 females)

Benign mixed tumor—very low incidence (Fischer 344/N: 1 reported in 1865 males 0.1%)

Malignant mixed tumor—very low incidence (Fischer 344/N: 1 reported in 1868 males 0.1%)

Pancreatic duct adenoma—very low incidence (Fischer 344/N: 1 reported in 1868 males 0.1%)

Parathyroid gland

Cyst—occasionally present.

Fibrosis, interstitial—occasional finding.

Hyperplasia, focal—occasional, sometimes multiple finding (Sprague–Dawley: 559 males 2.7%, 550 females 0.7%) (Sprague–Dawley: 2000 males 14%, 2000 females 1.9%).

Hyperplasia, diffuse—frequently occurs secondary to severe renal disease, a common disease in aged rats; high incidence in OFA–ICO rat is associated with severe renal disease and results in fibrous osteodystrophy.

Thymic rests

Tumors

Adenoma—low incidence

(Fischer 344/N: 1303 males 0.5%, 1328 females 0.2%)

(Sprague–Dawley: 559 males 2.7%, 550 females 0.7%)

Penis

Inflammation—incidence variable; wounds are common.
 Tumors: squamous cell carcinoma—2 reported in 51,230 Fischer 344/N rats.

Pericardium

Hyperplasia, papillomatous—a reactive hyperplasia that sometimes occurs with inflammation
 Inflammation—associated with inflammation of adjacent cardiac and mediastinal tissues
 Tumors—associated with neoplasia of adjacent tissues

Peripheral nerve (peripheral nervous system)

Radiculoneuropathy (radicular myelinopathy, degenerative myelopathy)—common degenerative finding in several rat strains associated with increased age; characterized by focal swelling of myelin sheaths or segmental demyelination and macrophages; most commonly affects the sciatic and tibial nerves, also the lumbar and ventral spinal nerve roots, and may involve dorsal spinal roots and spinal cord.

Tumors

Schwann cell tumors (schwannoma, benign or malignant; neurinoma, neurilemmoma, neurolemma, neurofibroma, neurofibrosarcoma, nerve sheath tumor, cystic sarcoma)—most common peripheral nerve tumors in rats; a granular cell variant has also been described.

Ganglioneuroma—rare tumor, more often observed in the adrenal gland than the spinal ganglia.

Peritoneum

Usually, peritoneal lesions are the result of changes in the mesentery, underlying intestine, or adjacent peritoneal cavity (acute, chronic, granulomatous inflammation, fat necrosis, fibrosis) (*see also* Mesentery).

Hemorrhage (hemoperitoneum)—sporadic finding resulting from rupture of intra-abdominal blood vessels.

Hyperplasia, mesothelial—reactive hyperplasia of the mesothelium is common, can be marked, and must be differentiated from neoplasia.

Tumors

Mesothelioma (mesenchymal sarcoma)—common in Sprague–Dawley and Fischer rats, especially adjacent to the testes
 (Fischer 344/N: 1936 males 2.7%, 1983 females 0.1%)

(Sprague–Dawley: 2 reported in 585 males 0.3%)

Metastatic tumors—common, especially sarcomas such as histiocytic sarcoma (malignant histiocytoma) and lymphoma

Pharynx (*see* Oral cavity)

Pineal gland—normal gland may appear large and be mistaken for hyperplasia or a tumor.

Tumors have been reported but are very rare.

Pituitary gland

Atrophy—occasional finding.

Cyst, simple—occasional finding, thought to be remnants of hypophyseal cleft (Rathke's pouch); occurrence 3%.

Fibrosis—marked increases in connective tissue are considered normal in aged rats; focal fibrosis sometimes associated with iron pigment suggests healed lesions.

Hyperplasia, focal—common proliferative findings related to hormones, diet changes, stress, light exposure, and temperature effects; may precede neoplasia.

(Sprague–Dawley: 579 males 16.1%, 581 females 6.4%)

(Sprague–Dawley: 2000 males 5.6%, 2000 females 4.8%)

Tumors

Adenoma—common in several strains; adenomas may show considerable cellular pleomorphism and nuclear atypia.

Adenoma, pars distalis

(Fischer 344/N: 1868 males 22.8%, 1934 females 45.2%)

(Sprague–Dawley: 579 males 62.2%, 581 females 84.7%)

(Sprague–Dawley: 2000 males 37.4%, 2000 females 63.4%)

Adenoma, pars intermedia (Fischer 344/N: 1868 males 0.2%, 1934 females 0.1%)

Carcinoma (adenocarcinoma)—very low incidence; diagnosis should be made only if clear infiltration of the surrounding brain is evident.

Carcinoma, pars distalis

(Fischer 344/N: 1868 males 2.3%, 1934 females 3.7%)

(Sprague–Dawley: 2000 males 3.1%, 2000 females 6.4%)

Craniopharyngiomas, malignant—rare tumors composed of squamous epithelium

(Fischer 344/N: 1 reported in 1898 males 0.1%)

(Sprague–Dawley: 579 males 0.2%, none in 581 females)

Pituitary, pars nervosa: glioma (Fischer 344/N: 2 reported in 1934 females 0.1%)

Pleura

Usually, pleural lesions are the result of changes in the underlying lung or adjacent mediastinum and thorax.

Tumors, mesothelioma (mesenchymal sarcoma)—very rare in Sprague–Dawley and Fischer rats; in contrast to the peritoneal serosa, especially testes (*see* Peritoneum).

Prepuce—inflammation, sporadic

Tumors—very rare

Keratoacanthoma—2 reported in 51,230 Fischer 344/N rats

Squamous cell carcinoma—4 reported in 51,230 Fischer 344/N rats

Preputial gland

Atrophy—occasional finding involving the acinar epithelium in aging rats.

Cyst—minor cystic changes commonly accompany atrophy. Degeneration, cystic—common finding in aged males, combination of changes including atrophy of acinar cells, fibrosis, distention of ducts by secreted material, and limited degrees of inflammation.

Enlargement—frequently observed grossly.

Hyperplasia, focal—occasional finding of the acinar epithelium in aging rats; sometimes cystic.

Hyperplasia, squamous cells of duct epithelium—often accompanies suppurative inflammation or abscess.

Inflammation (adenitis)—common findings that increase with age; acute adenitis characterized by accumulations of neutrophils that vary in severity from minimal to ulceration and abscess formation; chronic adenitis consists of focal and diffuse infiltrations by lymphocytes and occasional plasma cells with various proportions of neutrophils, macrophages, foreign body giant cells, and fibrosis.

Tumors—usually arise from acinar or ductal epithelium

Adenoma—low incidence (Fischer 344/N: 1936 males 3.8%)

Carcinoma—low incidence (Fischer 344/N: 1936 males 3.2%)

Prostate gland

Atrophy—characterized by simple squamous epithelium and dilatation with accumulation of proteinaceous secretions.

Cyst, mucinous—associated with chronic inflammation and can be mistaken for a carcinoma.

Dilatation (ectasis)—usually associated with atrophy.

Edema—observed in the interstitium associated with inflammation and may represent an agonal change.

Hyperplasia, epithelial—reactive hyperplasia is an occasional finding associated with inflammation; focal hyperplasia of the acinar epithelium is common in aged Sprague–Dawley rats; groups of 60–70 reported with range of 0.0%–43.3% (Sprague–Dawley: 580 males 9.7%) (Fischer 344/N: 1038 males 10.3%).

Inflammation (prostatitis)—both acute and chronic inflammatory changes are frequently present in aged rats; associated with inflammation of coagulating glands, seminal vesicles, bulbourethral glands, and preputial glands; leukocytic foci usually occur in 14% of young males (dorsal lobe, Fischer 344/N: 1383 males 70.4%; ventral lobe, Fischer 344/N: 1038 males 9.4%).

Suppurative—usually associated with inflammation of seminal vesicles, coagulation glands, urinary bladder, or kidneys (pyelonephritis).

Chronic—chronic inflammation, necrosis, and fibrosis commonly occur as small foci in aged rats, may be accompanied by mucinous cysts.

Squamous metaplasia—occasional finding accompanying chronic inflammation and epithelial hyperplasia.

Tumors

Adenoma—very low incidence

(Fischer 344/N: 1862 males 0.6%)

(Sprague–Dawley: 1 reported in 580 males 0.2%)

Carcinomas (adenocarcinoma)—occur occasionally in certain strains of aging rats, especially ACI/SegHap BR rats; rare in Fischer 344/N strain; actual incidences may be higher than historical data indicate because carcinomas are usually reported in the dorsal lobe that was not routinely examined histologically:

(Fischer 344/N: none reported in 3736 males; 10 reported in 51,230 Fischer 344/N rats)

(Sprague–Dawley: none reported in 580 males)

Lipoma (Sprague–Dawley: 1 reported in 580 males 0.2%).

Sarcoma—malignant histiocytomas and various soft-tissue tumors occur occasionally in the tissues around and in the prostate (Fischer 344/N: 1 leiomyosarcoma and 1 sarcoma, unspecified, reported in 51,230 rats).

Salivary gland (sublingual, mucous; parotid, serous; submandibular or submaxillary, mixed)

Atrophy—occasional finding, with loss of acinar tissues and dilatation of ducts lined by flat epithelium; glandular tissue may be replaced by fat, and mild chronic inflammation may be present.

Calculi (concretion, mineralization)—mineral deposits are occasionally found within the ducts.

Fibrosis—occasional finding associated with inflammation.

Inflammation (adenitis)—acute inflammation of the salivary gland is usually associated with virus infections, the most common being sialodacryoadenitis virus that also affects the lacrimal and Harderian glands, rat parvovirus, and cytomegalovirus.

Inflammation, focal—small foci of chronic inflammation and fibrosis are sporadic findings in otherwise-normal salivary glands.

Tumors—spontaneous tumors are very rare:

Adenoma—low incidence.

Incidence after gavage with corn oil vehicle (Fischer 344/N: 1 reported in 1914 males 0.1%, 3 reported in 1934 females 0.2%).

Adenocarcinoma—very low incidence (Fischer 344/N: 1 reported in 1895 males 0.1%).

Carcinoma, primary (Sprague–Dawley: 1 reported in 582 males 0.2%).

Squamous cell carcinoma—rare primary tumor; must be distinguished from infiltrating carcinomas.

Fibrosarcoma—very low incidence (Fischer 344/N: 1 reported in 1895 males 0.1%).

Sarcoma—very low incidence; the most common sarcoma in Sprague–Dawley rats is the undifferentiated, cystic sarcoma; also termed malignant schwannoma (Fischer 344/N: 2 reported in 1895 males 0.1%).

Seminal vesicles

Atrophy—common finding associated with diffuse testicular atrophy

Dilatation, cystic—common

Inflammation—common

Chronic—most common in interstitium

Suppurative—usually associated with prostate, testes, urinary bladder, or kidney inflammation (pyelonephritis)

Tumors

Adenoma—very low incidence (Fischer 344/N: 1 reported in 1949 males 0.1%)

Adenocarcinoma—very rare (Fischer 344/N: 1 reported in 51,230 rats)

Carcinosarcoma—very low incidence (Fischer 344/N: 1 reported in 1936 males 0.1%)

Skeletal muscle

Atrophy—occasional focal finding accompanied by inflammation in association with spontaneous degeneration of peripheral nerves in aged rats

Inflammation, chronic (myositis)—sporadic foci of lymphoid cells; sometimes associated with atrophy

Tumors—very infrequent in rats

Lipoma (Fischer 344/N: 2 reported in 1949 males 0.1%)

Liposarcoma (Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)

Fibrosarcoma—very low incidence (Fischer 344/N: 1 reported in 1936 males 0.1%)

Neurofibrosarcoma—very low incidence (Fischer 344/N: 1 reported in 1936 males 0.1%)

Hemangiosarcoma—very low incidence (Fischer 344/N: 1 reported in 1950 females 0.1%)

Rhabdomyosarcoma—very low incidence; must be differentiated from histiocytic sarcoma (Fischer 344/N: 1949 males 0.2%, 1983 females 0.1%)

(Sprague–Dawley: 1 reported in 584 females 0.2%)

Sarcoma, unspecified (NOS) (Fischer 344/N: 2 reported in 1949 males 0.1%)

Skin

Acanthosis (papillomatosis)—thickening of the squamous cell layers; sporadic finding, usually with inflammation.

Alopecia (hair loss)—sporadic finding, usually associated with other conditions including vices; patchy hair loss is most common; usual occurrence in young rats, 5%–9%.

Atrophy—occasional finding of the epidermis, sebaceous glands, and hair follicles, possibly associated with nutritional or hormone factors.

Edema—subcutaneous edema is occasionally observed as a result of a variety of causes including trauma, inflammation, nutritional imbalances, or an agonal change.

Epidermal cyst (inclusion cyst)—occasional finding of squamous cysts filled with keratin; sometimes accompanied by granulomatous inflammation (Sprague–Dawley: 585 males 1.7%, 584 females 0.3%).

Epidermoid cyst—occasional finding of squamous cysts containing hair follicle and sebaceous gland

epithelium; sometimes accompanied by granulomatous inflammation.

Fat necrosis—sporadic finding in the subcutaneous tissue; usually follows trauma or inflammation and is accompanied by granulomatous inflammation with macrophages, foreign body giant cells, fatty acid and cholesterol crystals, necrosis, and fibrosis.

Hyperkeratosis—thickening of the keratin (horny) layers; sporadic finding, usually with inflammation.

Inflammation—sporadic findings; usually related to trauma of the jaw, legs, feet, and tail; vary in severity and duration including focal ulceration of the epidermis, acute or acute necrotizing to chronic inflammation of the dermis and subcutis, and sometimes formation of abscesses and fibrosis; usual occurrence in young rats, males 4%; females 4%.

Necrosis—usually associated with inflammation and trauma.

Necrosis, gangrenous—dry gangrene involving the tail (ring tail) is associated with low-humidity environment.

Polyp, fibroepithelial—benign tumorlike lesions consisting of a fibrovascular stroma covered by normal-appearing epidermis; considered to be hyperplastic lesions.

Tumors, epidermal

Apocrine adenocarcinoma (Sprague–Dawley: 1 reported in 585 males 0.2%).

Basal cell adenoma (tumors)—low incidence (Fischer 344/N: 1936 males 0.7%, 1950 females 0.5%)

Basal cell carcinoma—low incidence (Fischer 344/N: 1936 males 0.7%, 1983 females 0.3%)

(Sprague–Dawley: 2 reported in 585 males 0.3%, 1 reported in 584 females 0.2%)

Basal cell epithelioma (Sprague–Dawley: 1 reported in 585 males 0.2%)

Inverted papilloma—the inverted papilloma forms a shallow cavity or depression usually filled with keratin and may be a type of keratoacanthoma.

Keratoacanthoma—low incidence; characterized by masses of basal and squamous cells with excessive keratin formation either within a crater or as one or more superficial projections.

(Fischer 344/N: 1936 males 1.6%, 1983 females 0.3%)

(Sprague–Dawley: 585 males 7.9%, 584 females 0.7%)

Sebaceous gland adenoma—very low incidence (Sprague–Dawley: 2 reported in 585 males 0.3%)

Sebaceous gland carcinoma (adenocarcinoma)—(Sprague–Dawley: 3 reported in 584 females 0.5%)

Squamous cell carcinoma—low incidence (Fischer 344/N: 1936 males 0.9%, 1983 females 0.3%)

(Sprague–Dawley: 585 males 0.9%, 584 females 0.5%)

- Squamous cell papilloma—low incidence
(Fischer 344/N: 1936 males 1.4%, 1983 females 0.3%)
(Sprague–Dawley: 585 males 2.1%, none in 584 females)
- Trichoepithelioma
(Fischer 344/N: 1936 males 0.3%, 1983 females 0.1%)
- Tumors, subcutaneous tissue
- Hemangioma (angioma, hemangioendothelioma)—very rare; some are believed to be congenital vascular malformations, not true neoplasms (Fischer 344/N: 2 reported in 1949 males 0.1%).
- Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—very low incidence
(Fischer 344/N: 1 reported in 1936 males 0.1%, 1 reported in 1983 females 0.1%)
(Sprague–Dawley: 1 reported in 585 males 0.2%)
- Cystic sarcoma (*see* Nerve sheath tumor, malignant)—undifferentiated sarcomas with a distinctive cystic appearance are occasionally observed in the subcutaneous tissue, salivary gland, uterus, mesentery, retroperitoneal tissues, and female genital tract; also termed malignant schwannoma.
- Dermatofibroma—superficial benign fibrous histiocyoma.
- Fibroma—low incidence
(Fischer 344/N: 1936 males 5.2%, 1983 females 1.3%)
(Sprague–Dawley: 585 males 1.9%, 584 females 0.3%)
- Fibrosarcoma—low incidence
(Fischer 344/N: 1936 males 1.3%, 1983 females 1.1%)
(Sprague–Dawley: 585 males 1.2%, 584 females 0.3%)
- Fibrous histiocyoma, malignant (malignant histiocytosis, extraskeletal giant cell tumor, fibrous histiocyoma, histiocyoma, one type of histiocytic sarcoma) (Fischer 344/N: 1936 males 0.2%, 1983 females 0.1%).
- Hemangiopericytoma—very low incidence (Sprague–Dawley: 1 reported in 584 females 0.2%).
- Hibernoma (brown fat tumor)—very rare (Sprague–Dawley: one has been reported).
- Histiocytic sarcoma—rare in Fischer 344/N.
- Lipoma—low incidence
(Fischer 344/N: 1936 males 0.4%, 1983 females 0.1%)
(Sprague–Dawley: 585 males 1.2%, 584 females 0.7%)
- Liposarcoma—very low incidence; may have features of a malignant hibernoma
(Sprague–Dawley: 1 reported in 584 females 0.2%)
- Lymphosarcoma (epitheliotropic lymphoma)—rare.
- Myxoma and myxosarcoma—very rare; existence is debated; usually considered as a form of fibroma or as undifferentiated sarcomas.
- Nerve sheath tumor, malignant (malignant schwannoma, neurilemmoma, cystic sarcoma)
(Fischer 344/N: 1936 males 0.3%, 1983 females 0.2%)
(Sprague–Dawley: 2 reported in 585 males 0.3%, 2 reported in 584 females 0.3%)
- Neurofibroma—low incidence (Fischer 344/N: 2 reported in 1936 males 0.1%)
- Neurofibrosarcoma—very low incidence (Fischer 344/N: 1936 males 0.4%, 1983 females 0.3%)
- Sarcoma, unspecified (undifferentiated sarcoma, sarcoma, NOS; mesenchymoma; *see* Cystic sarcoma)
(Fischer 344/N: 1936 males 0.5%, 1983 females 0.3%)
(Sprague–Dawley: 1 reported in 584 females 0.2%)
- Spinal cord (also *see* Brain)
- Cysts, epidermal—occasional congenital finding has been reported in 2.5% of CDF strain rats with lower frequencies in Lewis and Wistar HH stains.
- Radiculoneuropathy (radicular myelinopathy, degenerative myelopathy)—degeneration of the peripheral and spinal nerves in aged rats, may be accompanied by degeneration in the spinal cord and cauda equina; characterized by demyelination, swelling of axon sheaths and astrocytes, infiltration by macrophages, and, in advanced cases, mineralization.
- Pigmentation, lipofuscin—accumulates in neurons of aged rats as normal aging change.
- Tumors—rare (*see* Brain)
- Astrocytoma, malignant—(Sprague–Dawley: 2 reported in 585 females 0.3%)
- Oligodendroglioma, malignant—(Sprague–Dawley: 1 reported in 585 females 0.2%)
- Malignant schwannoma (Fischer 344/N: 1 reported in 1936 males 0.1%)
- Spleen
- Angiectasis—vascular dilatation usually focal and associated with fibrosis or scarring; sporadic.
- Atrophy—lymphocyte depletion is common in aged rats; may occur as a nonspecific reaction to stress or severe weight loss.
- Congestion—common finding; may be agonal related to the mode of death.
- Extramedullary hematopoiesis—only occurs in rats when under various stimuli when additional hematopoiesis is required; frequently observed in female rats having mammary gland fibroadenomas, especially if large and ulcerated (Sprague–Dawley: 4000 males and females 6%).
- Fibrosis—occasional finding of increased collagenous connective tissue.
- Hamartoma (*see* Nodular hyperplasia).
- Inflammation—rare; usually associated with inflammation extending from adjacent abdominal organs and peritoneum.
- Hyperplasia, erythroid—increased erythroid precursors are associated with anemia, hemorrhage, or erythrocytic destruction.
- Hyperplasia, lymphoid—low incidence, usually associated with systemic stimuli (Sprague–Dawley: 4000 males and females 6.2%).
- Hyperplasia, myeloid—increased immature granulocytic precursors associated with systemic inflammatory conditions.

Hyperplasia, nodular—occasional finding of well-defined, round, pale, or white nodules composed of a mixed population of mature lymphoid cells; may be hamartomas; must be differentiated from malignant lymphoma.

Hyperplasia, stromal—proliferation of mesenchymal cells associated with splenic trabeculae and marginal zones.

Pigmentation—iron-positive pigment (hemosiderin) is a normal finding in aged rats; in young rats, hemosiderin is generally more prominent in females than males; increased hemosiderin is associated with some forms of anemia, hemolysis, and increased erythrocyte destruction; lipofuscin or ceroid pigmentation occurs in some strains, including Fischer 344/N.

Mineralization—sometimes observed with necrosis; may be confused with pigmentation.

Tumors—blood vessel tumors and sarcomas are most common:

- Hemangioma (angioma, hemangioendothelioma)—rare (Fischer 344/N: 1 reported in 1906 males 0.1%) (Sprague–Dawley: 1 reported in 585 males 0.2%)
- Hemangiosarcoma (angiosarcoma, hemangioendothelioma)—low incidence (Fischer 344/N: 1906 males 0.3%, 1961 females 0.1%) (Sprague–Dawley: 2 reported in 585 males 0.3%)
- Fibroma—low incidence (Fischer 344/N: 1 reported in 1906 males 0.1%).
- Fibrosarcoma—low incidence (Fischer 344/N: 1 reported in 1348 males 0.07%).
- Fibrous histiocyctoma—low incidence (Fischer 344/N: 1 reported in 1348 males 0.07%).
- Histiocytic sarcoma (malignant histiocyctoma)—Sprague–Dawley: 4000 males and females 0.2%.
- Leiomyoma—very rare (Fischer 344/N: 1 reported in 1906 males 0.1%).
- Leiomyosarcoma (Fischer 344/N: 2 reported in 1348 males 0.15%, 1 reported in 1961 females 0.1%).
- Leukemia, myelogenous (myeloid or granulocytic) (also *see* Lymph node) (Sprague–Dawley: 585 males 0.3%, 585 females 0.2%) (Sprague–Dawley: 4000 males and females 0.8%).
- Leukemia, mononuclear cell (large granular lymphocytic leukemia, monocytic leukemia, lymphosarcoma)—appears to arise in the spleen and secondarily involves the bone marrow and lymph nodes; common in Fischer 344 rats (Fischer 344/N: 1936 males 33.6%, 1983 females 20.2%); incidence is reportedly decreased in males by gavage with corn oil vehicle (Fischer 344/N: 1949 males 17.1%, 1950 females 19.3%).
- Lipoma (Fischer 344/N: 1 reported in 279 males 0.36%).
- Lymphosarcoma (malignant lymphoma)—frequently involves the spleen as well as lymph nodes; uncommon in Fischer 344/N strain (also *see* Lymph node) (Sprague–Dawley: 4000 males and females 0.5%).
- Sarcoma, unspecified (NOS)—low incidence (Fischer 344/N: 1906 males 0.4%, 1961 females 0.1%).

Stomach (glandular)

Atrophy—focal atrophy with fibrosis and dilatation of the gastric glands is occasionally observed in aged rats and has been reported to be very common in some Sprague–Dawley colonies.

Congestion—common finding resulting as an agonal change or accompanying inflammation.

Cyst—common to have cystic dilatation of glands accompanied by atrophy, chronic inflammation, hyperplasia, or mineralization (calcification).

Ectopic pancreatic tissue—sporadic finding.

Erosion (*see* Ulcer).

Diverticulum—rare finding.

Hyperplasia, glandular (adenomatous hyperplasia, hypertrophy, hypertrophic gastritis, proliferative gastritis, hyperplastic gastropathy)—occasional finding in aged rats of hyperplastic and cystic glands; thickened lamina propria, focal chronic inflammation, and dilated submucosal blood vessels

(Sprague–Dawley: 2000 males 1.3%, 2000 females 1%)

Inflammation (gastritis)—acute and chronic active inflammations are common findings, often accompanying erosions or ulcerations; scattered inflammatory cells can occur in the mucosa without ulceration or necrosis; occasionally crypts contain leukocytes (crypt abscess); chronic inflammation with lymphoid cells is occasionally observed in the submucosa (Sprague–Dawley: 2000 males 1.2%, 2000 females 1.2%).

Mineralization (calcification)—focal aggregates of calcium are found in the gastric glandular epithelium; metastatic calcification occurs in the glandular mucosa associated with severe renal disease and parathyroid hyperplasia; dystrophic calcification also occurs in the smooth muscles (Sprague–Dawley: 2000 males 3.4%, 2000 females 1%).

Necrosis—superficial necrosis (erosion) and deep necrosis (ulceration) are common findings (*see* Ulcer) (Sprague–Dawley: 2000 males 4.2%, 2000 females 4%).

Ulcer—erosions and ulcers (focal necrosis) are sporadic low incidence findings that can result from a variety of causes including cold, restraint, stress, shock, vascular occlusion, and feeding schedule; accompanied by acute or chronic active inflammation (acute or chronic necrotizing inflammation) (Sprague–Dawley: 2000 males 6.5%, 2000 females 5%).

Tumors—rare in rats

Adenoma—very rare (Fischer 344/N: none in 1912 males and 1955 females)

Adenocarcinoma (carcinoma)—very low incidence (Fischer 344/N: 1 reported in 1912 males 0.1%)

Fibrosarcoma—very low incidence (Fischer 344/N: 1 reported in 1924 males 0.1%)

Neurofibrosarcoma—very low incidence (Fischer 344/N: 1 reported in 1912 males 0.1%)

Sarcoma—very low incidence (Fischer 344/N: 1 reported in 1955 females 0.1%)

Stomach (nonglandular) (*see* Forestomach)

Subcutaneous tissue (*see* Skin)

Testes

Arteritis (periarteritis, polyarteritis nodosa)—occasional finding; usually in association with other organs.

Atrophy of seminiferous tubules—common finding occurs in a variety of patterns and frequencies; may be associated with a relative increase in Sertoli cell numbers; may be observed at the first year of life; usual incidence rate in young males is 2%; pressure atrophy is often observed adjacent to large tumors, especially interstitial cell tumors such as in Fischer (F344) rats (Sprague–Dawley: 2000 males 12.3%).

Atrophy, focal—atrophy associated with aging usually begins as a focal lesion and may involve one or both testes; term focal may be used for lesions affecting less than 50% of the seminiferous tubules of the testis.

Atrophy, diffuse—usually affects most or the whole of one or both testes; term may be used for lesions affecting 50% or more of the seminiferous tubules; often leaving only Sertoli cells in the tubules; tubules are usually smaller with thin walls; giant cells are associated with degenerated epithelium; mineralized deposits may be present.

Degeneration, cystic—an age-related change characterized by the presence of fluid-filled cystic spaces; usually associated with focal atrophy.

Dilatation—atrophy must be differentiated from simple dilatation of the seminiferous tubules.

Edema—must be differentiated from a common artifact resembling central edema.

Inflammation, spermatic granulomas—occasional.

Interstitial (Leydig) cell hyperplasia—common finding especially in strains with high frequencies of interstitial cell tumors; may precede tumor formation; usually associated with testicular atrophy (Sprague–Dawley: 580 males 3.4%)

Mineralization, focal—common.

Necrosis, hemorrhagic, focal—reported as a result of parvovirus infections in adult rats.

Tumors

Adenomatoid tumor (mesothelioma)—rare tumor observed in epididymis, capsule, or spermatic cord of Sprague–Dawley rats (Sprague–Dawley: 2 malignant mesotheliomas reported in 585 males 0.3%) (*see* Peritoneum)

Hemangioma (angioma, hemangioendothelioma)—uncommon (Fischer 344/N: none reported in 1910 males)

Interstitial (Leydig) cell adenoma—most common testicular tumor; often bilateral, incidence increases with age; high incidence in several rat strains, especially in Fischer (F344) (Fischer 344/N: 1910 males 87.8%) (Sprague–Dawley: 585 males 6.5%) (Sprague–Dawley: 2000 males 6.7%)

Interstitial (Leydig) cell carcinoma—uncommon to very low incidence depending on criteria used for diagnosis; up to 10% of interstitial cell tumors

(Fischer 344/N: 1 reported in 1350 males 0.07%)

(Sprague–Dawley: 1 reported in 585 males 0.2%)

Leukemias and lymphomas—occasionally infiltrate the testes

Malignant histiocytoma (histiocytic sarcoma)—occasional finding

Seminoma—very rare (Fischer 344/N: none reported in 1910 males)

Sertoli cell tumor—very rare (Fischer 344/N: none reported in 1910 males)

Teratoma—very rare (Fischer 344/N: none reported in 1910 males)

Thymus

Atrophy—normal physiological depletion of lymphocytes (involution) and proliferation of epithelial cells begins at sexual maturity and proceeds under the influence of sex and strain; the atrophy is accelerated by various infections and other diseases, stress, and trauma; as the lymphoid tissue of the cortex decreases, the Hassall's corpuscles become more prominent (Sprague–Dawley: 4000 males and females 10.7%).

Cyst—sporadic findings in both cortex and medulla; some cysts can be large and lined by columnar or flattened, squamous epithelium; some are derived from remnants of branchial endoderm, the thymopharyngeal duct (Sprague–Dawley: 4000 males and females 1.7%).

Ectopic parathyroid tissue—uncommon finding within the thymic capsule.

Hyperplasia, cystic (cystic change, epithelial hyperplasia)—Hassall's corpuscles sometimes become cystic and hyperplastic; more common in Sprague–Dawley females.

Hyperplasia, lymphoid—rare finding in rats (Sprague–Dawley: 4000 males and females 1.4%).

Hyperplasia, tubular (epithelial hyperplasia)—frequent finding especially in rats older than 2 years of age, especially Fischer 344/N.

Tumors

Histiocytic sarcoma (malignant histiocytoma) (Sprague–Dawley: 4000 males and females 0.3%)

Leukemia, myelogenous (myeloid or granulocytic) (Sprague–Dawley: 4000 males and females 0.3%)

Lymphosarcoma—rare tumors in Fischer 344/N rats that arise in the thymus and extend into adjacent mediastinal tissues without splenic involvement (Sprague–Dawley: 4000 males and females 0.4%)

Thymoma—rare tumors composed of epithelial cells with various amounts of lymphoid cells; often large tumors resembling adenocarcinomas; may be infiltrative in the mediastinum (Fischer 344/N: 1484 males 0.2%, 1597 females 0.1%)

Thymoma, benign—Sprague–Dawley: 530 males 0.4%, 537 females 0.6%

Thyroid gland

Amyloidosis—very rare observation in rats; usually associated with C cell (medullary) carcinomas.

Cyst—common finding; usually arises from ultimobranchial duct remnants; single large follicles are normal in mature rats and should not be designated as cysts.

Thymic rests—ectopic thymus occurs in 2%–3% of young rats.

Hyperplasia, C cell, focal—common (frequent in Long-Evans strain)

(Sprague–Dawley: 583 males 8.6%, 581 females 13.3%)

(Sprague–Dawley: 2000 males 6.2%, 2000 females 7.8%)

Hyperplasia, follicular cell, focal—common (Sprague–Dawley: 583 males 1.9%, 581 females 0.5%).

Inflammation—focal acute inflammation and chronic inflammations are occasionally observed; chronic lymphocytic thyroiditis is sometimes reported in some strains.

Pigmentation (hemosiderosis)—iron-positive pigment can be observed occasionally, usually in the large dilated follicles.

Tumors

C cell tumors—common finding (Fischer 344/N: 1904 males 12%, 1938 females 11%)

C cell adenoma—low incidence

(Fischer 344/N: 1904 males 7.7%, 1938 females 8.0%)

(Sprague–Dawley: 583 males 6.5%, 581 females 5.9%)

(Sprague–Dawley: 2000 males 3.5%, 2000 females 2.9%)

C cell (medullary) carcinoma (adenocarcinoma)

(Fischer 344/N: 1904 males 3.8%, 1938 females 3.4%)

(Sprague–Dawley: 583 males 1.9%, 581 females 0.5%)

(Sprague–Dawley: 2000 males 2.8%, 2000 females 2.4%)

Follicular cell adenoma—low incidence

(Fischer 344/N: 1904 males 0.7%, 1938 females 0.6%)

(Sprague–Dawley: 583 males 3.9%, 581 females 1.5%)

Follicular cell adenocarcinoma (carcinoma)—very low incidence

(Fischer 344/N: 1904 males 0.5%, 1938 females 0.4%)

(Sprague–Dawley: 583 males 2.2%, 581 females 1.4%)

Tongue

Inflammation—uncommon

Tumors

Squamous cell papilloma (Fischer 344/N: 3572 males 0.3%, 3570 females 0.4%)

Squamous cell carcinoma (Fischer 344/N: 1 reported in 3572 males 0.03%)

Schwann cell tumors (schwannoma, benign or malignant; nerve sheath tumor, malignant) (Sprague–Dawley: 1 reported in 525 males 0.2%)

Tooth

Dysplasia

Malocclusion—acquired and genetic predisposition

Tumors—very low incidence

Odontoma (Fischer 344/N: 2 reported in 1936 males 0.1%)

Trachea

Inflammation, acute or chronic (tracheitis)—frequent findings of small foci of inflammatory cell infiltrates; submucosal aggregates of lymphoid cells are common; may be involved as a part of a variety of either upper or lower respiratory tract infections

Tumors: adenocarcinoma (Fischer 344/N: 1 reported in 1934 males 0.1%)

Ureter (*see* Kidney)

Urethra

Inflammation, acute or chronic (urethritis)—sporadic; most inflammation in rats is usually associated with inflammation of the bladder and prostate, or pyelonephritis.

Mucous plugs—occasional (*see* Urinary bladder).

Tumors—rare

Transitional cell carcinoma (adenocarcinoma) (Fischer 344/N: 1 reported in 1936 males 0.1%)

Urinary bladder

Inflammation, acute or chronic (cystitis)—sporadic; infiltrations of small numbers of inflammatory cells are common; most inflammation in rats is usually associated with inflammation of the prostate or pyelonephritis; the brown Norway rat (BN/Bi) has usually high frequency, probably secondary to ureteric or bladder tumors or urolithiasis (Sprague–Dawley: 2000 males 2.5%, 2000 females 0.7%).

Dilatation—frequently observed at necropsy; important only if obstruction is present.

Hyperplasia, urothelial—focal (nodular) or diffuse; uncommon; usually a reactive response of the transitional cell epithelium associated with chronic inflammation or calculi; hyperplasia, especially papillary, must be differentiated from neoplasia (Sprague–Dawley: 2000 males 2.8%, 2000 females 0.7%).

Metaplasia, squamous—occasionally observed associated with chronic inflammation in aged rats; reported to occur as a result of vitamin A-deficient diets.

Mineralization (calcification, calculi, stones)—calculi are usually a sporadic finding; some strains have higher frequencies than others that may relate to diet and metabolism; calculi have been reported to be related to increased tumor incidences.

Mucous plugs—mucinous, hyaline amorphous, or proteinaceous material occasionally present; usually considered incidental finding unless there is evidence of urinary obstruction; must be differentiated from calculi; other small rounded eosinophilic (proteinaceous) droplets may be observed in normal bladders that seem to arise from the urothelium.

Tumors—spontaneous occurrence is very low:

Polyp (Sprague–Dawley: 1 reported in 581 females 0.2%)

Transitional cell papilloma—very low incidence (Fischer 344/N: 1858 males 0.2%, 1932 females 0.2%)

Transitional cell carcinoma (adenocarcinoma)—very low incidence
(Fischer 344/N: 2 reported in 1891 males 0.1%, 1 reported in 1932 females 0.1%)
(Sprague–Dawley: 581 males 0.2%, 581 females 0.2%)

Uterus

Adenomyosis—rare finding of endometrial glands within the myometrium of aged rats.

Atrophy—thinning of the endometrium and myometrium increases with age.

Cysts (cystic change)—cystic dilated glands are common in the endometrium.

Dilatation—common; uterine horns normally become distended with intraluminal fluid including mucus during the estrous cycle (proestrus); usual incidence is 14% in young female rats; must be differentiated from hydrometra.

Fibrosis—increased amounts of collagen in endometrium and myometrium; accompanies cystic endometrial hyperplasia.

Hydrometra—common; excessive dilatation of the uterus in aged rats.

Hyperplasia, cystic endometrial—regularly results after prolonged estrogen exposure as occurs with ovarian follicular cysts and granulosa cell tumors.

Hyperplasia and hypertrophy, stromal cell—usually associated with cystic endometrial hyperplasia.

Hypertrophy, portio vaginalis uteri or cervical—sporadic finding in old rats that could be misinterpreted as a leiomyoma or fibroma.

Inflammation (acute endometritis, chronic endometritis, myometritis, pyometra)—inflammation is sporadic in the endometrium; often occurs in association with vaginal inflammation; usually accompanies obstructive or neoplastic lesions; most inflammatory changes are associated with bacterial or mycoplasma infections.

Metaplasia, squamous—usually associated with pyometra.

Polyps, inflammatory—focal hyperplasia resulting from inflammation; must be differentiated from benign neoplastic polyps.

Pyometra—suppurative inflammation (*see* Inflammation).

Tumors—occasional finding in aged rats

Adenoma (polypoid adenoma or glandular polyp)—very low incidence
(Fischer 344/N: 1966 females 0.2%)

Adenocarcinoma—not common; usually polypoid
(Fischer 344/N: 1966 females 0.4%)
(Sprague–Dawley: 1 reported in 572 females 0.2%)

Endometrial stromal polyp—common benign tumor in uterine horns; can be predominantly stromal, glandular, or mixed; some polyps are probably not true neoplasms but focal hyperplasia
(Fischer 344/N: 1966 females 21.4%)
(Sprague–Dawley: 1 reported in 572 females 0.2%)

Endometrial stromal sarcoma—common malignant tumor

(Fischer 344/N: 1966 females 1.1%)

(Sprague–Dawley: 1 reported in 572 females 0.2%)

Fibroma—occasional finding; must be differentiated from leiomyoma

(Fischer 344/N: 3 reported in 1934 females 0.2%)

(Sprague–Dawley: 1 fibroma of cervix reported in 261 females 0.4%)

Fibrosarcoma—occasional finding; must be differentiated from leiomyosarcoma

Granular cell tumor (Fischer 344/N: 1 reported in 1934 females 0.1%)

Hemangioma (angioma, hemangioendothelioma)

(Fischer 344/N: 2 reported in 1966 females 0.1%)

(Sprague–Dawley: 1 fibroma of cervix reported in 261 females 0.4%)

Hemangiosarcoma (angiosarcoma, hemangioendotheliosarcoma)—rare (Fischer 344/N: none reported in 1966 females)

Leiomyoma—occasional tumors that may extend into the vagina; low incidence

(Fischer 344/N: 3 reported in 1966 females 0.2%)

(Sprague–Dawley: 2 reported in 584 females 0.3%)

Leiomyosarcoma—low incidence

(Fischer 344/N: 4 reported in 1966 females 0.2%)

(Sprague–Dawley: 1 reported in 584 females 0.2%)

(Sprague–Dawley: 1 fibroma of cervix reported in 261 females 0.4%)

Lipoma—occasional tumors in Sprague–Dawley rats

Malignant schwannoma (*see* Sarcoma, cystic) (Fischer 344/N: 2 reported in 1966 females 0.1%)

Sarcoma, cystic—undifferentiated sarcoma with cystic areas that resembles cystic sarcoma of subcutis; also considered to be a form of malignant schwannoma

Sarcoma, unspecified (NOS) (Fischer 344/N: 3 reported in 1966 females 0.2%)

Squamous cell carcinoma

(Fischer 344/N: 2 reported in 1966 females 0.1%)

(Sprague–Dawley: 2 reported in 584 females 0.3%)

Vagina

Inflammation, suppurative—occasional finding that is usually associated with changes of the uterus; must be differentiated from normal cyclical changes of metestrus and diestrus.

Dilatation (imperforate vagina)—occasional finding with massive cystic dilatation that may be mistaken for a cyst.

Fibrosis—increased deposition of collagen reported in the walls with increasing age.

Tumors

Fibroma

(Fischer 344/N: 1 reported in 1950 females 0.1%)

(Sprague–Dawley: 1 reported in 520 females 0.2%)

Granular cell tumor (Fischer 344/N: 1 reported in 1983 females 0.1%)

Leiomyosarcoma (Fischer 344/N: 1 reported in 1950 females 0.1%)

Sarcoma (Fischer 344/N: 1 reported in 1983 females 0.1%)

Squamous cell carcinoma—occasional finding in aged Sprague–Dawley rats (Sprague–Dawley: 2 reported in 520 females 0.4%)

Squamous cell papilloma (Fischer 344/N: 1 reported in 1950 females 0.1%)

Zymbal's gland (*see* Ear)

GROSS AND HISTOPATHOLOGIC FINDINGS IN CONTROL LABORATORY DOGS^{48–79}

The dog preferred for regulatory toxicity studies is the beagle. These animals are bred for laboratory use, have known parentage, are generally free of disease, have a convenient size, and are easy to handle. The following findings are primarily reported for the laboratory beagle:

Adrenal gland, cortex

The width of the various zones in the cortex can vary considerably. The zona glomerulosa may be thin or appear to be absent in places. Occasionally, medulla cells can be observed in the cortex.

Degeneration—fatty degeneration was reported. This degeneration must be distinguished from normal variation in vacuolation.

Inflammation—focal inflammation occurs infrequently. Mild focal inflammation was reported in 5 (8%) of 647 dogs.

Hyperplasia—focal cortical or nodular hyperplasia was reported in 19 (3%) of 647 dogs.

Vacuolation of cortical cells varies greatly from animal to animal.

Adrenal gland, medulla

Occasionally, cortical cells can be observed in the medulla.

Aorta (*see* Blood vessel)

Artery (*see* Blood vessel)

Blood

Anemia is an absolute decrease in the packed cell volume, hemoglobin concentration, and red blood cell count. The clinical signs of anemia include pale mucous membranes, weakness, fatigue, labored breathing upon exertion, rapid heart rate, and altered heart sounds such as a murmur. Hemolytic diseases result in anemia accompanied by icterus (jaundice), a yellowish pigmentation of the mucous membranes associated with deposition of bile pigment, especially bilirubin. Anemia with icterus can follow extensive hemorrhage or excessive lysis of red blood cells (hemolysis).

Bacteremia—a persistent presence of bacteria in the blood is associated with canine brucellosis (*Brucella canis*).

Blood vessel (major)

Collection of blood samples from the cephalic vein very rarely results in sufficient injury to cause lesions.

Congenital patent ductus arteriosus was observed grossly in 1 (0.1%) of 1000 dogs.

Degeneration, aorta—focal medial degeneration was reported in 1 (0.1%) of 647 dogs.

Inflammation (arteritis) occurred in 2% of young beagles. Extramural periarteritis of the coronary artery was reported in 1 (0.1%) of 647 dogs.

Mineralization of the aorta occurred occasionally in the aortic media near the base of the heart in 2%–3% of young beagles.

Bone

Fractures of ribs are unusual gross findings and were observed grossly in 1 (0.1%) of 1000 dogs.

Chondrodystrophy of costochondral junction was reported in 1 (0.1%) of 647 dogs.

Brain

Degeneration—degenerating axis cylinders may be observed as spherical, eosinophilic granular structures in the medulla oblongata, pons, or anterior cervical spinal cord, most common in the gracilis tract and nucleus. These structures apparently have no neurological or pathological significance.

Gliosis—focal proliferation or small subependymal collections of glial cells are observed usually around the anterior parts of the lateral ventricles. Hemorrhage is occasionally seen in brains of untreated laboratory dogs. Brain hemorrhage was reported in 2 of 37 (5%) untreated beagle dogs.

Hydrocephalus is characterized by the dilatation of ventricles or subarachnoid space as a result of abnormal accumulation of cerebrospinal fluid (CSF). When the dilatation is limited to the ventricles, the usual form, it is termed internal hydrocephalus. When it affects the subarachnoid space, it is termed external hydrocephalus. When both locations are affected, it is termed communicating hydrocephalus. Extensive dilatation of the lateral ventricles may be found during trimming of brains from dogs that showed no neurological signs. It is the most frequently reported lesion of the nervous system of dogs, is probably congenital in origin, and is common in laboratory beagles. Hydrocephalus was observed grossly in 14 (1%) of 1000 dogs. A spongeliike alteration has been reported in the brain tissue adjacent to ventricular hydrocephalus.

Inflammation (encephalitis, meningitis, meningoencephalitis)—chronic focal meningitis is common. It occurred in 44 (7%) of 630 dogs on 33 studies. Focal encephalitis is uncommon and was reported in 5 (0.8%) of 630 dogs.

Inflammation, granulomatous—*Toxocara* granulomas have been reported in the brain.

Ear—middle and inner ears are not routinely examined during toxicology studies; therefore, spontaneous lesions in

untreated control beagles were not reported in the literature reviewed. Lesions of the external ear or pinna are reported under skin.

Epididymis

Inclusion bodies—intranuclear, eosinophilic, and PAS positive inclusions are normal findings in the epididymal epithelium of dogs and have unknown significance.

Pigment—normal epididymal cells have granular, yellow to yellow-brown pigment.

Inflammation (epididymitis)—epididymitis is uncommon. It was reported in 1 (0.3%) of 326 males. Lymphocytic epididymitis is characterized by infiltrates of lymphocytes. These lesions are usually associated with lymphocytic orchitis and thyroiditis in laboratory beagles. Inflammation can also be the result of canine brucellosis (*B. canis*) (see Testes).

Inflammation, granulomatous—spermatogenic granulomas (spermatocele granulomas) result from injury to epididymal tubules containing sperm.

Esophagus

Dilatation—megaesophagus is characterized by a grossly dilated and flaccid esophagus. This is a congenital condition that can result from a persistent right aortic arch or more commonly the result of an apparent neuromuscular developmental disorder.

Dilatation, esophageal gland ducts—slightly dilated ducts may be seen in an otherwise-normal esophagus.

Hypertrophy—swollen and thickened walls often associated with reflux esophagitis.

Inflammation (esophagitis)—an erosive and ulcerative esophagitis can result from repeated reflux of stomach contents.

Eye—microscopic lesions of eyes in laboratory dogs are uncommon. No microscopic lesions were observed in 630 beagles. However, ophthalmoscopic and slit-lamp biomicroscopic examinations reveal a variety of changes in laboratory beagles. Persistent hyaloid vessel remnants, vitreous floaters, vitreous filaments, atapetal fundi, tigroid fundi, tapetal hyperreflectivity, and old hemorrhage or scars have been reported in the eyes of beagles between 6 and 12 months of age.

Anemia results in pale mucous membranes (see Blood):

Blood vessels—hyaloid artery remnants persisted in 142 (26.7%) of 532 dogs examined. This is not considered to be a pathologic finding. The hyaloid artery usually regresses shortly after birth but at times fails to regress and remains attached to the posterior lens capsule.

Gland of the third eyelid—adjacent to the third eyelid is a compound alveolar gland that may become neoplastic in older dogs, resulting in a red growth protruding from under the third eyelid at the medial canthus of the eye.

Protrusion or prolapse of the third eyelid occurs in young beagles. This is often associated with the inflammation of bulbar conjunctiva and superficial glandular tissue. The ducts of the gland may become dilated

and contain leukocytes. The conjunctival tissue is usually hyperplastic.

Lymphoid cell infiltration of the third eyelid and gland has been reported.

Icterus (jaundice) is a yellowish pigmentation of the mucous membranes associated with deposition of bile pigment, especially bilirubin. Icterus can be the result of extensive hemorrhage, lysis of red blood cells (hemolysis), gallbladder or bile duct obstruction, and liver diseases affecting the bile ducts.

Inflammation, conjunctiva—conjunctivitis associated with sawdust bedding and dust occurred in 4% of young beagles.

Inflammation, granulomatous—*Toxocara* granulomas have been reported in the retina and choroids of young beagles.

Eye, cornea

Opacities—corneal opacities were observed from 3 to 8 years of age.

Inflammation (keratitis) has been reported in beagles between 6 and 12 months of age and at 8 years of age. Superficial keratitis or focal corneal opacities were observed in 35 (6.6%) of 532 dogs examined.

Eye, lens

Prominent posterior lens sutures were the only ophthalmoscopic change reported in 86 laboratory beagles less than 3 years of age. From 3 to 8 years, prominent posterior lens sutures, nuclear opacities, and capsular (anterior, posterior, and peripheral) opacities were observed. Other studies of beagles between 6 and 12 months of age reported additional findings of posterior cortical opacities, posterior polar opacities, lenticular sheen, vacuoles in the cortex, and zones of discontinuity.

Lenticular sheen—a yellowish sheen is observed when light is reflected as fluorescence from the lens media during ophthalmoscopic examinations. This is usually associated with senile or aging changes in the lens; however, it can occur in younger animals. Lenticular sheen was observed in 120 (22.6%) of 532 dogs examined.

Posterior cortical opacities or water clefts associated with the posterior Y sutures were observed in 54 (10.1%) of 532 dogs examined. These opacities are believed to be due to a greater proportion of water content in the cortex.

Zones of discontinuity are the result of refractive changes in normal stratification of the lens and may be related to metabolic disturbances in lens fiber growth. They have been considered to be presenile changes and occurred in 32 (6.0%) of 532 dogs examined.

Eye, retina

Atrophy—thinning and absence of tapetal cells (see Pigment).

Degeneration, cystoid—peripheral retinal cystoid degeneration consists of single or multiple microcysts within the retina at or near the ora serrata. Although a common

change in older beagles, it occurs in younger animals.

At 8 years of age, this lesion had an 85% incidence rate.

Dysplasia—focal retinal dysplasia occurs occasionally and includes retinal folds, retinal rosettes, focal absence of retinal cells, and blending of nuclear layers.

Fibrosis—scarring of the retinal was observed at 8 years of age.

Mineralization—calcified bodies occur between the epithelial layer and the choroid.

Pigmentation—pigment cells have been observed in the rod and cone layer. Pigment clumps are normal findings in tapetum lucidum that are characterized by focal pigmentation of the retinal pigment epithelium overlaying the tapetum. Tigroid fundi represent a form of pigment hypoplasia in the retinal pigmented epithelium. This absence of pigment allows the choroidal vessels to be seen as irregular networks of orange-red broad ribbons. In atapetal fundi, the tapetum lucidum is missing. Other tapetal aberrations include focal or linear areas of hyperreflectivity, old focal hemorrhages, or scars. Tapetal aberrations and pigment clumps were observed in 25 (4.7%) of 532 dogs examined. The pigmentation findings are considered structural variants of basically normal eyes.

Eye, vitreous

Vitreous floaters or asteroid bodies are small relucet bodies that are composed of calcium salts suspended in normal vitreous. These bodies move when the eye moves. Vitreous filaments result from vitreous hemorrhage or remnants of the posterior vascular capsule that are observed as strands of fibrinous residues. Vitreous floaters and filaments occurred in 53 (10.0%) of 532 dogs examined.

Gallbladder

Hyperplasia—cystic mucinous hyperplasia at early stages may be present.

Lymphoid foci—individual lymphoid follicles may be prominent.

Gallstones—fine brown or black crystalline gallstones were reported.

Heart

A variety of congenital and acquired findings involve the heart of laboratory beagles.

Congenital findings included pulmonary stenosis, patent ductus arteriosus, and valvular telangiectasis. Pulmonary stenosis was observed grossly in 1 (0.1%) of 1000 dogs.

Degeneration—degenerative changes of the myocardium must be interpreted with caution because many artifacts result from handling and contraction of myofibrils during fixation.

Fatty infiltration of the myocardium was reported in 13 of 37 (35%) untreated beagle dogs.

Fibrosis is a component of chronic inflammation, occurs throughout the heart, and may be associated with

mineralization (calcification). Fibrosis of the myocardium may be a healed lesion of parvovirus infection. Focal myocardial fibrosis and mineralization was reported in 2 (0.1%) (<1%) of 647 dogs.

Hemorrhage—postmortem imbibition of blood pigments can stain the intima of both the heart and blood vessels to grossly resemble hemorrhage.

Inflammation, chronic—chronic valvular fibrosis was observed grossly in 27 (3%) of 1000 dogs. Chronic focal pericarditis was reported in 1 (0.1%) of 647 dogs.

Inflammation, granulomatous—*Toxocara* granulomas have been reported in the myocardium. Granulomas were reported in 2 (0.3%) of 647 dogs.

Inflammation, myocardium—myocarditis is infrequent. Focal myocarditis was reported in 4 (0.6%) of 647 dogs. Parvovirus causes myocarditis in puppies and recently weaned dogs but is largely controlled by vaccination. Healed lesions may be present in older dogs.

Inflammation, coronary artery—periarteritis has been reported.

Leukocyte foci were observed within the myocardium in 1% of young beagles.

Valvular angiectasis (telangiectasis, hematocyst, congenital hematoma) is occasionally observed involving atrioventricular valves, usually the right. They appear grossly as small blood cysts and consist microscopically of blood-filled endothelial-lined spaces. Valvular telangiectasis was observed grossly in 4 (0.4%) of 1000 dogs.

Valvular endocardiosis is considered the most frequent incidental finding in the heart and is characterized by swollen, soft, and glistening septal cusps of the right atrioventricular valve.

Nematodes (heartworms, *Dirofilaria immitis*)—heartworms were not reported in laboratory beagles but are reported in experimental dogs from other sources. Heartworm microfilaria is transmitted by infected mosquitoes and is a risk to dog colonies. Infected dogs with early or moderate disease tire quickly during exercise. In advanced disease, the lungs can have large emboli, thrombi, and pneumonia; the right heart is enlarged with large adult nematodes. Clinical signs are consistent with right heart failure.

Intestine

Congestion—mucosal blood vessels are commonly congested as a result of digestive processes, but can be secondary to inflammation, and occur as agonal changes.

Cysts—mucoepithelial cysts of the small intestine have been reported.

Diverticulum—Meckel's diverticulum, a congenital defect in the small intestine, has been reported.

Hernia—intestines can protrude through congenital defects in the abdominal wall. Congenital hernias involving intestines were observed grossly in 5 (0.5%) of 1000 dogs.

Hyperplasia, lymphoid—mild hyperplasia of individual or aggregates of lymph follicles may be seen in the

lamina propria of untreated animals. These hyperplastic follicles usually correlate with the presence of nodules observed at necropsy.

Inflammation, large intestine (cectitis; colitis)—mild focal cecitis or colitis was reported in 25 (4%) of 647 dogs.

Inflammation, granulomatous—granulomas were observed in the large intestine of 1%–3%, and in the small intestine of 2%–4% of young beagles. Small intestine granulomas were reported in 6 (0.9%) of 647 dogs. *Toxocara* granulomas have been reported in the intestine.

Inflammation, small intestine (enteritis)—catarrhal enteritis is common. It was reported in 148 (23%) of 647 dogs. Parvovirus causes acute enteritis in weaned dogs but is largely controlled by vaccination.

Intussusceptions have been observed in laboratory dogs.

One dog was affected grossly in 1000 (0.1%) dogs.

Metazoan parasites

Protozoa—Giardiasis (*Giardia* sp.) is important in young dogs. The disease can result in intermittent or chronic diarrhea, which may persist for several months accompanied by malabsorption of nutrients with reduced growth rate, weight loss, dull hair coat, and other clinical signs.

Coccidiosis (*Isospora* spp.) in young dogs can result in diarrhea and dehydration. Infections with *Isospora bigemina* can cause intestinal hemorrhage.

Nematodes (roundworms: nematodiasis)—nematodes are common and occurred in 23%–28% of young beagles. Most are observed in the small intestine. Usually, they are ascarids.

Ancylostomiasis (*Ancylostoma* spp.) Hookworms usually affect young dogs and can result in pale oral membranes, anemia, and reduced growth rate. If the infection is heavy, black tarry feces can be observed. Ancylostomiasis was reported in 1 (3%) of 37 untreated beagle dogs.

Ascariasis—the common large roundworms of dogs belong to the genera, *Toxocara* spp. A few ascarids (*Toxocara canis* or *Toxascaris leonina*) are often observed in small numbers free in the small intestine lumen of laboratory dogs. Ascarids were observed grossly in 165 (17%) of 1000 dogs.⁵⁰ Ascarids were reported in 2 of 37 (5%) untreated beagle dogs.

T. canis larvae migrate through the liver and lungs before reaching the small intestines. When migrating larvae die or molt, they can incite chronic or granulomatous inflammation (granulomas) characterized by the presence of eosinophils. Some larvae reach other tissues where they can cause additional inflammatory lesions. The most common sites for granulomas are the mesenteric lymph nodes and lungs; other sites include the renal cortex.

Strongyloides (*Strongyloides stercoralis*)—strongyloides are small threadlike nematodes found in the small intestines. They were reported in 9 (1%) of 647 dogs.

Trichuriasis—the whipworm of the dog, *Trichuris vulpis*, is uncommon in laboratory beagles. They are found in the cecum and colon. Trichuriasis was reported in 7 of 37 (19%) in a colony of beagle dogs.

Tapeworms—cestodes are uncommon and are observed grossly in 8 (0.8%) of 1000 dogs. The most common tapeworm is the “cucumber seed” *Dipylidium caninum*, which is usually asymptomatic but can result in diarrhea and anal pruritus.

Kidney

Agensis—unilateral renal agenesis is a congenital failure of one kidney to develop. It is not detected during routine physical examinations but occasionally occurs in laboratory beagles. The ureter may be missing. The developed kidney is usually about twice normal size. Unilateral renal agenesis was observed grossly in 4 (0.4%) of 1000 dogs.

Hydronephrosis occurs occasionally in dogs. It was observed grossly in 7 (0.7%) of 1000 dogs.

Inclusions, intranuclear—rectangular or cubic acidophilic inclusions are frequent findings in the nuclei of renal tubular epithelial cells of dogs. These crystalline acidophilic intranuclear inclusions are commonly observed in nuclei of cells lining proximal and distal tubules. They are identical in appearance to those seen in hepatocytes.

Inflammation (nephritis)—focal nonspecific inflammation is common and can occur as leukocyte foci, or be the result of bacterial emboli. Leukocyte foci occurred in 1%–4% of young beagles. Minor focal nonspecific inflammation was reported in 76 (12%) of 647 dogs. Focal embolic nephritis was observed in 1 of the 647 dogs.

Inflammation, glomerulus (glomerulitis, glomerulonephritis, glomerulosclerosis)—glomerulonephritis is characterized by inflammation involving the glomeruli. Glomerulitis and glomerulonephritis were reported in 2 (0.3%) of 647 dogs. Glomerulosclerosis—a progressive lesion of the glomerulus that has also been termed progressive intercapillary glomerulosclerosis is seen as early as 6 months of age. It is characterized by thickened basement membranes and increased mesangial matrix. Local or diffuse mesangial proliferation and thickened, wrinkled glomerular basement membranes are not usual findings in clinically healthy laboratory beagles. Periglomerular sclerosis may also be present. The severity of intercapillary sclerosis increases with time, and intermittent or persistent proteinuria may occur.

Inflammation, granulomatous—cortical *Toxocara* granulomas are common, and were reported in 4% of young beagles. Granulomas were reported in 11 (2%) of 647 dogs. Cortical granulomas were reported in 2 of 37 (5%) untreated beagle dogs.

Inflammation, interstitium (chronic interstitial nephritis)—interstitial nephritis was observed in 6% of young beagles. Chronic interstitial nephritis was reported in 6 (0.9%) of 647 dogs.

Inflammation, pelvis (pyelitis, pyelonephritis)—pyelitis was observed in 4%–7% of young beagles. Pyelonephritis is a nephritis that results from inflammation arising in the pelvis that spreads upward to involve the medulla and, subsequently, the cortex. Pyelitis or pyelonephritis was reported in 12 (2%) of 647 dogs.

Lipidosis, glomerular—occasionally, one or more lobes of the glomerulus will be filled by large foam cells, which react positively when stained for fat. This lesion has unknown significance.

Mineralization (calcification) is very common and occurred in 69%–74% of young beagles. Mineralization usually occurs as microcalculi, small foci of basophilic deposits in collecting tubules of the renal medulla, or papilla in almost 50% of both sexes. Microcalculi were reported in 312 (48%) of 647 dogs. Renal collecting tubule calcification was reported in 6 of 37 (16%) untreated beagle dogs.

Tumors—a renal cell carcinoma was observed grossly in 1 (0.1%) of 1000 dogs.

Liver

Gross and microscopic findings are very frequently reported in the liver.

Cysts—subcapsular cysts have been observed in untreated dogs.

Fatty change (fatty degeneration, fatty metamorphosis, lipidosis)—focal fatty change was observed grossly in 78 (8%) of 1000 dogs. Focal cytoplasmic vacuolization of hepatocytes can occur adjacent to the base of the hepatic ligaments (*see* Tension lipidosis).

Hepatocyte cytoplasmic vacuolation—this can result from normal storage of glycogen. Large amounts of glycogen are expected after a meal; however, extensive cytoplasmic vacuolation from glycogen can be present after fasting overnight.

Hepatocyte vacuolar degeneration—this may be a form of fatty change.

Hyperplasia, bile duct—hyperplasia occurs with, and without, portal inflammation. Focal bile duct hyperplasia was uncommon. It was reported in 2 (0.3%) of 647 dogs.

Inclusions, intracytoplasmic—acidophilic globular inclusions occur in the cytoplasm of hepatocytes of dogs. They stain positive with PAS reagents, but are not glycogen. They consist of proteinaceous material and bound lipids.

Inclusions, intranuclear—rectangular, cubic, or rhomboid acidophilic (hyaline) inclusions are frequent findings in the nuclei of hepatocytes of dogs. They are commonly termed acidophilic intranuclear inclusions and appear to be composed of protein. They were reported in 2 (0.3%) of 647 dogs.

Inflammation, bile duct (cholangitis)—cholangitis is usually associated with necrosis or other inflammation of the liver parenchyma and was not reported as a primary finding in control beagles.

Inflammation, veins—eosinophilic phlebitis and periphlebitis are suggestive of a hypersensitivity reaction to migrating parasites. Focal phlebitis was very common and occurred in 409 (63%) of 647 dogs.

Inflammation, portal—mild portal inflammation with, and without, bile duct hyperplasia is common. It was reported in 50 (8%) of 647 dogs.

Inflammation, granulomatous—microgranulomas are the most common lesion observed microscopically in the liver of young beagles. These lesions consist of small focal collections of histiocytes (macrophages or mononuclear cells), lymphocytes, an occasional neutrophil, and a few degenerate hepatocytes. Their cause is unknown but some may be related to parasite migration, especially *Toxocara* spp. Granulomas were observed in 2%–4% of young beagles. They were reported in 23 (4%) of 647 dogs.

Leukocyte foci are very common and occurred in 47%–60% of young beagles.

Lipidosis (*see* Fatty change).

Necrosis—hepatocyte necrosis is observed in several forms and is associated with several degenerative and inflammatory lesions. Focal necrosis and inflammation was very common in the liver. They were reported in 423 (65%) of 647 dogs.

Pigmentation—small brown pigment granules are seen in both hepatocytes and Kupffer cells. The pigment is usually either lipofuscin or hemosiderin, or both. Individual hepatocytes and Kupffer cells may have both pigments. Bile pigments may be present if biliary stasis is present.

Tension lipidosis—focal subcapsular lipidosis and necrosis seen near the hilus at the base of hepatic ligaments result from tension on the liver capsule and anoxia of adjacent hepatocytes.

Lung

Inflammation is very common in the lungs and occurs in a large variety of forms including perivascularitis, peribronchiolitis, pleuritis, subpleural fibrosis, endobronchiolitis, bronchopneumonia, interstitial pneumonia, and various granulomas. Most of the pulmonary pathology observed in untreated laboratory beagles has been associated with infections by lungworms, the nematode *Filaroides hirthi*.⁵⁸ Some inflammatory lesions may be due to inhaled sawdust bedding. Gavage accidents resulting in lung lesions are very rare in dogs.

Anthraxis (*see* Pigmentation).

Atelectasis—collapsed alveoli are usually associated with inflammation or pressure from fluid in the thorax.

Bronchopneumonia (*see* Inflammation, bronchi).

Congestion—active congestion is usually a component of inflammation but also occurs with poor circulation from heart diseases and during agonal events. Pulmonary hyperemia was reported in 2 of 37 (5%) untreated beagle dogs.

Emphysema—distended or ruptured alveoli are uncommon.

Helminthiasis (*see* Nematodes)

Hemorrhage in the lung is an uncommon finding.

Pulmonary hemorrhage was reported in 1 of 37 (3%) untreated beagle dogs.⁵⁴

Hyperemia (*see* Congestion).

Inflammation, alveoli (alveolitis)—fibrosing alveolitis, a fibrous thickening of the alveolar walls, occurred in 1%–3% of young beagles.

Inflammation, interstitium (pneumonitis, interstitial pneumonia)—interstitial pneumonia is common and occurred in 22%–28% of young beagles. Focal interstitial pneumonia was reported in 111 (17%) of the 647 dogs. Granulomatous pneumonitis was reported in 13 of 37 (35%) untreated beagle dogs.

Inflammation, bronchi and bronchioles—bronchopneumonia is unusual in laboratory dogs because of vaccination programs against canine distemper. Canine distemper is a virus that predisposes dogs to bacteria, which can cause pneumonia. Bronchopneumonia was reported in 3 of 37 (8%) untreated beagle dogs. Bronchopneumonia caused by *Mycoplasma* spp. has been reported. Localized bronchitis and bronchiolitis occurred in 2%–8% of young beagles. Perivascularitis and peribronchiolitis were reported in 329 (51%) of 647 dogs. Subpleural fibrosis and endobronchiolitis occurred in 139 (21%) of the 647 dogs. Pleuritis was reported in 1 (3%) untreated beagle dogs.

Inflammation, granulomatous—granulomas are common. Granulomas were reported in 6%–7% of young beagles and in 75 (12%) of 647 dogs on 39 studies. Both lungworms and migrating nematode larvae cause granulomas in the lungs. Granulomas are frequently observed as small tan, green, or gray subpleural nodules that are associated with the lung worm, *F. hirthi*. *Toxocara* granulomas have also been reported. Vascular microgranulomas may result following emboli from intravenous injection sites. They were reported in 8 (1%) of the 647 dogs. Cholesterinic granulomas occur less frequently. One dog of the 647 had a cholesterinic granuloma. Granulomatous pneumonitis was reported in 13 of 37 (35%) untreated beagle dogs.

Leukocyte foci are common and occurred in 17%–18% of young beagles.

Nematodes (nematodiasis)—living nematodes were observed in 2%–6% of young beagles. *See* Inflammation, granulomatous, in the preceding text.

Pigmentation (anthracosis) is unusual because most laboratory dogs are not exposed to dusty environments. Anthracosis was reported in 19 of 37 (51%) untreated beagle dogs from one colony.

Lymph node (unspecified, NOS)

Mandibular, mesenteric, and medial retropharyngeal lymph nodes are the most frequently processed for histopathology.

Congestion of the mesenteric lymph nodes is common and can have a normal physiological origin. Congestion must be distinguished from hemorrhage.

Hyperplasia, lymphoid—nonneoplastic proliferation of lymphoid cells is frequently observed resulting in increased numbers of lymphocytes and related cells.

Hyperplasia is usually associated with inflammation and infections. It must be distinguished from early neoplastic lesions.

Plasmacytosis—a prominent increase in plasma cells has been reported.

Inflammation (lymphadenitis)—generalized lymphadenitis is associated with canine brucellosis (*B. canis*).

Inflammation, granulomatous—granulomas are most common in the mesenteric lymph nodes. They are often the result *Toxocara*. They were observed in 21%–27% of young beagles, and in 16 (2%) of 647 dogs from 39 studies.

Tumors—lymphosarcoma affecting the lymph nodes has been reported in beagles less than 1 year of age.

Mammary gland

Hyperplasia—mammary nodules rarely occur in non-treated, control, female dogs. In a 7-year study, 40 mammary nodules were palpated in 7 of 18 control bitches. Most were transient observations. At the time of necropsy, nine were present in six animals. Five of the nine nodules were lobular or intraductal hyperplasia. Two were benign mixed mammary tumors. The other two were non-mammary lesions. In another 4-year study, nodules were palpable in 4 of 20 control bitches between 21 and 24 months, but none were present at necropsy.

Inflammation (mastitis) is rare; one animal (<1%) with mastitis was reported in 321 female beagles.

Tumors—neoplasms of mammary glands are unusual in beagles less than 7 year of age. During life-span observations of 1343 beagles, 476 (70.8%) of 672 females and 2 (0.3%) of 671 males had one or more mammary neoplasms. Classification and detailed descriptions of canine mammary tumors can be found in Moulton.

Benign mixed mammary tumors were observed in 2 (11%) of 18 control female beagles during a 7-year study.

Mouth (*see* Oral cavity)

Nerve (*see* Peripheral nerve)

Oral cavity (palate, nasopharynx)

Papillomas (warts) are benign epithelial tumors caused by papovavirus that are usually observed in young dogs and can spontaneously disappear. Oral papillomas may be found on the lips, inside the cheeks, and on the tongue, palate, and pharynx. The gums are usually not affected. Oral papillomas were observed grossly in 2 (0.2%) of 1000 dogs.

Oral mucosa

Anemia results in pale mucous membranes (*see* Blood).

Icterus (jaundice) is a yellowish pigmentation of the mucous membranes associated with deposition of bile pigment, especially bilirubin. Icterus can be the result of extensive hemorrhage, lysis of red blood cells (hemolysis), gallbladder or bile duct obstruction, and liver diseases affecting the bile ducts.

Ovary

Cyst—clear cysts are occasionally observed in the vicinity of the ovary in young adult beagles. These have been reported as parovarian cysts. This is not a specific diagnosis. Parovarian cysts were observed grossly in 2 (0.4%) of 499 females.

Pancreas

Degeneration—cytologic alteration has been reported in acinar and islets cells. Edema was reported.

Infiltrates—various types of leukocytes have been observed infiltrating the pancreatic tissues.

Inclusions, intracytoplasmic—ovoid acidophilic inclusions often containing basophilic particles in clear spaces may occur in acinar cells of dogs. These inclusions are ultrastructurally similar to dense ribosomal autophagic vacuoles.

Inflammation (pancreatitis)—chronic focal pancreatitis was reported in 8 (1.2%) of 647 dogs.

Inflammation, granulomatous—*Toxocara* granulomas have been reported in the pancreas (*see* Liver).

Parathyroid gland

Cysts are uncommon in the parathyroid. Small cysts may be found within or near the parathyroid apparently arising from the duct connecting the thymus and parathyroid primordia. They are usually multiloculated, lined by cuboidal to columnar epithelium, often ciliated, and contain densely eosinophilic material. Cysts occurred in 1% of young beagles.

Hyperplasia of the parathyroid cells was reported in 7 (1%) of 647 dogs.

Penis

Inflammation of glans penis (balanitis)—mild inflammation of the glans penis, and prepuce (balanoposthitis), is common in dogs.

Peripheral nerve

Renaut bodies are normal structures within nerves that could be mistaken for nerve infarction or necrotizing angio-pathic neuropathy. They appear as cylindrical, loosely textured, whorled, cell-sparse structures in nerves.

Degeneration—spontaneous degenerative lesions of the peripheral nerve may be seen in the beagle. While commonly considered an aging change, occasionally, “digestion chambers” and “myelin bubbles” are seen in the sciatic nerves of young beagles.⁷

Pharynx (*see* Oral cavity)

Pituitary gland

Cysts are very frequently observed in the pituitary. Most of these are remnants of the craniopharyngeal duct and are located at the periphery of the pars tuberalis and distalis. Usually the cysts are microscopic, lined by ciliated cuboidal to columnar epithelium, and contain mucin. Cysts occurred in 24%–26% of young beagles and in 44 (7%) of 647 dogs. Pituitary cysts were reported in 3 of 37 (8%) untreated beagle dogs.

Inflammation, granulomatous—granulomas have been reported associated with *Toxocara*. Granuloma was reported in 1 (0.1%) of 647 dogs.

Prepuce

Inflammation (posthitis)—mild inflammation of the prepuce accompanied by inflammation of the glans penis (balanoposthitis) is common in dogs. It can result in a slight mucopurulent preputial discharge.

Prostate

Atrophy—atrophy of the epithelium has been associated with chronic inflammation. It was reported in 7 (2%) of 326 males.

Hyperplasia, cystic—focal cystic hyperplasia was reported in 15 (5%) of 326 dogs.

Lymphoid or leukocyte foci occurred in 12% of young beagles¹⁹ (*see* Inflammation, lymphocytic).

Inflammation (prostatitis)—subclinical prostatitis is common in the dog. The inflammation is usually minimal in young adult beagles. Prostatitis was reported in 12% of young beagles and in 124 (38%) of 326 dogs from 39 studies. Some prostatitis may result from catheterization during collection of urine.

Inflammation, lymphocytic—lymphocytic prostatitis may consist of minor lymphocytic infiltrates (foci), up to large lymphoid aggregates with germinal centers. Large lesions are accompanied by interstitial fibrosis and epithelial atrophy (chronic prostatitis). Inflammation can also be the result of canine brucellosis (*B. canis*).

Salivary gland

Inflammation (sialoadenitis) is not usual. Mild focal sialoadenitis was reported in 35 (5%) of 647 dogs.

Skeletal muscle

Hernias are congenital weaknesses in the abdominal wall, usually located in the umbilical area, and contain only omental fat. These are infrequent findings. Congenital hernias involving intestines were observed grossly in 5 (0.5%) of 1000 dogs.

Inflammation, granulomatous—granulomas of *Toxocara* sp. occur in skeletal muscle but are unusual findings.

Skin

Skin lesions are quite common as a result of abrasions from concrete surfaces and caging, as well as from bite wounds caused by fighting.

Terminology for dermatohistopathology often uses a specialized vocabulary. Diagnostic criteria and illustrations of skin findings for laboratory dogs are well described by Hargis, Muller, and Yager and Scott.

Alopecia is commonly observed in laboratory dogs from a variety of causes; although some cases have unknown cause, most occur as complete, partial, diffuse, or circumscribed loss of hair without other skin changes over bony pressure points such as the elbow and hock. Alopecia that occurs at circumscribed, erythematous, and scaly areas near the eyes, at the commissures of the

lips, around the mouth, or on the forelegs is often the result of demodectic mange mites. *Demodex canis* or *follicularum* is a normal resident of the skin, inhabits the hair follicles and sebaceous glands, and is estimated to be present in 80% of animals in some colonies. Demodectic mites were reported in 1 (0.1%) of 647 dogs on 39 studies.

Callus (callosity)—this is a localized hyperkeratotic lesion from continued trauma of the skin over the bony pressure points where alopecia occurs. These areas may become ulcerated and infected resulting in pressure point granulomas.

Dermatosis—ear margin lesions observed as multiple irregular, soft, tan nodules on the margins of the pinnae characterized by hyperkeratosis and parakeratosis.

Furunculosis—extensive areas of inflammation are often the result of a penetrating or perforating folliculitis and can result from large demodectic mite populations.

Hyperkeratosis—increased amounts of keratin were not recorded as a primary finding.

Inflammation, skin (dermatitis)—dermatitis was observed in 4%–7% of young beagles.

Inflammation, hair follicle (folliculitis)—folliculitis and perifolliculitis are often associated with demodectic mites.

Parakeratosis—a form of hyperkeratosis with cell nuclei in the accumulated keratin was not recorded as a primary finding.

Tumors—neoplasia in the dog is most common between ages 6 and 14 years; however, tumors do occur in younger animals. A detailed description of canine tumors can be found in Muller and Moulton.

Skin tumors reported in beagles less than 2 years of age are rare.

Histiocytoma was reported in 1 (0.1%) of 647 dogs.

Mast cell sarcoma and sarcoma unspecified have also been reported.

Spinal cord

Degeneration—degenerating axis cylinders may be observed as spherical, eosinophilic granular structures in the medulla oblongata, pons, or anterior cervical spinal cord, most common in the gracilis tract and nucleus. These structures apparently have no neurological or pathological significance.

Inflammation (myelitis)—focal myelitis has been observed in the spinal cords of 2 (0.3%) of 647 dogs.

Inflammation, granulomatous—*Toxocara* granulomas have been reported in the spinal cord (cauda equina).

Spleen

Accessory spleens are occasionally observed in the gastrosplenic omentum. They can be congenital, but many appear to be acquired through traumatic rupture of the spleen. Accessory spleens were observed grossly in 1 (0.1%) of 1000 dogs. Angiectasis (telangiectasis) is localized dilatation of blood vessels that are observed as dark red blebs around the margin of the spleen. They

consist of blood-filled sinusoids that fail to empty during the splenic contraction associated with exsanguinations. They occurred in 7%–14% of young beagles.

Congestion (hyperemia) is commonly observed grossly, especially following euthanasia with barbiturates. It must be distinguished from active congestion associated with inflammation and secondary congestion associated with poor blood circulation. Splenic hyperemia was reported in 1 of 37 (3%) untreated beagle dogs.

Extramedullary hematopoiesis—the production of blood cells in the splenic pulp, especially the erythroid series, is uncommon in adult dogs. Splenic extramedullary hematopoiesis was reported in 3 of 37 (8%) untreated beagle dogs.

Fibrosis—the proliferation of fibrous connective tissue usually accompanies inflammation. Splenic fibrosis was reported in 1 (3%) of 37 untreated beagle dogs.

Hemorrhage—because of the blood flow and abdominal location, hemorrhage is common in the spleen. Hemorrhage must be distinguished from congestion. Splenic hemorrhage was reported in 1 (3%) of 37 untreated beagle dogs.

Hematomas are localized areas of hemorrhage and are infrequently observed. They were observed grossly in 2 (0.2%) of 1000 dogs.

Siderotic or siderofibrotic nodules can be small, localized, and nodular thickened areas on the capsular surface, larger irregular encrustations covering of extensive areas of the capsule, or nodules in the pulp. They are grossly yellow to grayish brown and commonly occur around the margin of spleens, or at the attachment of the gastrosplenic omentum in older dogs. Siderofibrotic nodules were observed grossly in 90 (9%) of 1000 dogs. They have also been termed Gandy–Gamna bodies. Microscopically, they consist of fibrotic foci that are often calcified and contain brown pigment (hemosiderin) and yellow pigment (hematoidin). They probably are the end result of hemorrhage and thrombosis.

Hemosiderosis—deposition of iron-containing pigment associated with the breakdown of red blood cells in splenic tissues is commonly associated with siderotic nodules.

Splenic hemosiderosis was reported in 17 of 37 (46%) untreated beagle dogs.

Hyperplasia, lymphoid—these are nonneoplastic proliferations of lymphoid cells, which result in increased numbers of lymphocytes and related cells and can cause enlargement of the spleen. Hyperplasia is usually associated with inflammation and infections. It must be distinguished from early neoplastic lesions.

Inflammation, granulomatous—granulomas have been associated with *Toxocara*, and were observed in 21%–27% of young beagles.

Reticulosis—this is a form of lymphoid hyperplasia with a predominance of reticuloendothelial or histiocytic cells.

Lymphosarcoma affecting the spleen and lymph nodes has been reported in beagles less than 1 year of age.

Stomach

Atrophy—atrophy of parietal cells may be associated with gastric spirillum-like bacteria.

Dilatation—dilatation of gastric glands may be associated with gastric spirillum-like bacteria.

Hyperplasia, lymphoid—lymphoreticular hyperplasia may be associated with large numbers of gastric spirillum-like bacteria that are commonly found within the lumen of gastric glands and in the intracellular canaliculi of parietal cells.

Inflammation (gastritis)—chronic gastritis is common in laboratory dogs and was reported in 58 (9%) of 647 dogs.

Inflammation, granulomatous—granulomas were reported in 1 (0.1%) of 647 dogs.

Lymphoid foci—lymphoid nodules or follicles are commonly present within the lamina propria, especially in the pyloric region.

Mineralization occurs as basophilic granules in the middle fundic mucosa in both sexes and affects 7%–10% of young beagles.

Testes

Atrophy—focal atrophy of seminiferous tubules occurred in 5% of young beagles and in 11 (3%) of 326 males.

Degeneration—testicular degeneration is frequently observed in adult dogs and may be focal or diffuse, unilateral or bilateral. Early lesions consist of a loss of germ cells that can appear as individual degenerated cells or as multinucleated giant cells. As degeneration progresses, more germinal cells are lost resulting in almost empty tubules lined by sustentacular (Sertoli) cells. Intratubular giant cells are observed in seminiferous tubules of 9 (3%) of 326 untreated young beagles.

Hyperplasia, interstitial cell—interstitial cell hyperplasia is rare in untreated beagles. It was reported in 1 (0.3%) of 326 dogs.

Inflammation (orchitis)—lymphocytic orchitis is characterized by diffuse, aggregated, or nodular infiltrates of lymphocytes. Germinal centers may be present. These lesions are usually associated with lymphocytic thyroiditis in laboratory beagles and are commonly accompanied by focal or diffuse degeneration and atrophy of seminiferous tubules. The epididymis may be involved. Inflammation can also be the result of canine brucellosis (*B. canis*).

Thymus—the amount of thymus varies considerably in short-term toxicology studies. Involution begins prior to sexual maturity.

Atrophy is uncommon in untreated beagles and must be distinguished from involution.

Ectopic thyroid occurred in 1% of young beagles.

Cysts occurred in 1% of young beagles.

Thyroid gland

Atrophy—follicular atrophy has been observed. Idiopathic thyroid atrophy is a progressive loss of follicular epithelium, with replacement by adipose cells. The lobes are affected unequally and if severely affected, may

be difficult to find at necropsy. Parafollicular cells are unaffected and can be seen in the adipose tissue.

Ectopic thymus occurred in 1%–2% of young beagles.

Cysts—cysts are common findings. Ultimobranchial duct cysts are most frequently observed in thyroid, they arise from remnants of the ultimobranchial body, and have a keratinized squamous epithelial lining. Cysts of the thyroid were observed in 1%–2% of young beagles.

Hyperplasia, C cell (parafollicular cells)—the numbers of C cells in young beagles vary widely with occasionally focal increases. C cell hyperplasia occurred in 8%–9% of young beagles.

Hyperplasia, follicular cell—follicular cell hyperplasia was reported infrequently.

Hyperplasia, lymphoid—lymphoid hyperplasia occurred in 2% of young beagles.

Inflammation—lymphocytic and unspecified thyroiditis have been reported in beagles. Lymphocytic thyroiditis appears to be immunologically mediated and have a familial occurrence in beagles. The lesions consist of multifocal to diffuse infiltrates of lymphocytes, plasma cells, and macrophages. Lymphoid nodules may be present. The thyroid follicles are usually small and may contain degenerate follicle cells, lymphocytes, and plasma cells. Thyroiditis was reported in 3 of 37 (8%) untreated beagle dogs.

Inflammation, granulomatous—granulomas associated with *Toxocara* have been reported.

Metaplasia, squamous—focal squamous metaplasia has been reported.

Tongue

Inflammation (glossitis) occurred in 1%–2% of young beagles.

Inflammation, granulomatous—granulomatous inflammation can be associated with fragments of sawdust bedding embedded in the tongue.

Papillomas (warts) are benign epithelial tumors caused by papovavirus that usually are observed in young dogs and can spontaneously disappear. *See* Oral cavity.

Tooth

Minor dental abnormalities are frequently observed. They can include missing permanent teeth (usually upper and lower premolars), retained deciduous teeth (usually canine), imperfect apposition, dental plaque (soft masses of bacteria and food), and dental calculus or tartar (mineralized plaque, usually discolored, most abundant next to orifices of salivary ducts).

Inflammation (gingivitis) is usually associated with plaque or tartar.

Trachea

Inflammation (tracheitis)—chronic tracheitis was reported in 11 (2%) of 647 dogs on 39 studies.

Urinary bladder

Inflammation (cystitis)—cystitis occurs infrequently in untreated beagles, predominantly in males. Some

cystitis may result from catheterization during collection of urine. Cystitis was reported in 27, 23 males and 4 females (4%), of 647 dogs.

Arteritis and periarteritis were present in 10 of 59 dogs with detrusor myopathy. Both sexes were equally affected.

Mineralization—calculi were observed grossly in the urinary bladder lumen of 5 (0.5%) of 1000 dogs. Calcification of the round ligament of the bladder, a remnant of the umbilical artery of the fetus, is an incidental finding in young beagles.

Myopathy—detrusor myopathy is characterized by degenerative lesions in the urinary bladder muscle tunic and was present in 59 of 449 (13%) young beagle dogs. Arteritis and periarteritis were frequently present. Both sexes were equally affected.

Urethra

Congestion of the mucosal wall can be prominent at necropsy. This normal physiological filling of capillaries and veins can be confused with hemorrhage.

Mineralization—urethral calculi are unusual and were observed grossly in 1 (0.1%) of 1000 dogs.

Uterus

Cyst—myometrial cysts have been reported in young beagles. One dog was affected in 499 females (0.2%).

Distension (dilatation) occurred in 11% of young beagles.

Inflammation (endometritis)—endometritis associated with canine brucellosis (*B. canis*) can result in abortion in the third trimester of pregnancy.

Zygomatic (orbital or dorsal buccal) gland is a salivary gland located at the apex of the orbit of the eye. It sometimes becomes abscessed and can cause the eye to protrude.

Organs or tissues that had no reported spontaneous lesions in the available references included anal gland, anal sac, bone marrow, circumanal glands, clitoris, ductus deferens, ear, lacrimal gland, larynx, mesentery, nose, oviduct, pancreatic islets, ureter, and vagina. The dog does not have seminal vesicles or bulbourethral glands.

Comments: For a general review of disease problems of laboratory dogs, especially those from random sources and those conditioned for research, refer to Ringler and Peter. Pick and Eubanks reported gross and microscopic findings for 49 random source dogs in North Carolina. This is not a comprehensive presentation of all pathology findings for dogs used in toxicology studies. Additional information and more detailed descriptions of canine diseases, parasitology, and pathology are available in a number of veterinary medical texts. See Aiello, Bonagura, Carter, Hettinger and Feldman, Jones, Jubb, Muller, Summer, and Urquhart.

REFERENCES

1. Sanders, B. J., Animal Histology Procedures of the Pathological Technology Section of the National Cancer Institute, HEW Publication No. (NIH) 72275 Superintendent of Documents, U.S. Government Printing Office, Washington, DC, 1972.

2. Luna, L. G., Ed., *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd edition, McGraw-Hill, New York, 1968, chap. 1.
3. Luna, L. G., *Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts*, American Histolabs, Inc., Gaithersburg, MD, 1992.
4. Sheehan, D. C. and Hrapchak, B. B., *Theory and Practice of Histotechnology*, 2nd edition, C. V. Mosby, Saint Louis, MO, 1980.
5. Hardisty, J. F. and Eustis, S. L., Toxicological pathology: A critical stage in study interpretation, in *Progress in Predictive Toxicology*, Clayson, D. B., Munro, I. C., Shubik, P., and Swenberg, J. A., Eds., Elsevier, New York, 1990, p. 41.
6. World Health Organization, *Principles and Methods for Evaluating the Toxicity of Chemicals. Part 1*, Environmental Health Criteria 6, World Health Organization, Geneva, Switzerland, 1978, p. 192.
7. Hardisty, J. F. and Boorman, G. A., National toxicology program pathology quality assurance procedures, in *Managing Conduct and Data Quality of Toxicology Studies*, Hoover, K. B., Baldwin, J. K., Velner, A. F., Whitmire, C. E., Davies, C. L., and Bristol, D. W., Eds., Princeton Scientific, Princeton, NJ, 1986, p. 263.
8. Haseman, J. K., Arnold, J., and Eustis, S. L., Tumor incidences in Fischer 344 rats: NTP historical data, in *Pathology of the Fischer Rat*, Boorman, G. A., Eustis, S. L., Elwell, M. R., Montgomery, C. A., and MacKenzie, W. F., Eds., Academic Press, New York, 1990, p. 555.
9. Seaman, W. J., *Postmortem Change in the Rat: A Histologic Characterization*, Iowa State University Press, Ames, IA, 1987.
10. Benirschke, K., Garner, F. M., and Jones, T. C., Eds., *Pathology of Laboratory Animals*, vols. I and II, Springer-Verlag, New York, 1978.
11. Bhatt, P. N., Jacoby, R. O., Morse, H. C., and New, A. E., *Viral and Mycoplasmal Infections of Laboratory Rodents*, Academic Press, New York, 1986.
12. Clapp, N. K., *An Atlas of RF Mouse Pathology: Disease Descriptions and Incidences*, Technical Information Center, Office of Information Services, United States Atomic Energy Commission, Oak Ridge, TN, 1973.
13. Cotchin, E. and Roe, F. J. C., *Pathology of Laboratory Rats and Mice*, Blackwell Scientific, Oxford, U.K., 1967.
14. Faccini, J. M., Abbott, D. P., and Paulus, G. J. J., *Mouse Histopathology*, Elsevier, New York, 1990.
15. Firth, C. H. and Ward, J. M., *Color Atlas of Neoplastic and Nonneoplastic Lesions of Aging Mice*, Elsevier, New York, 1988.
16. Firth, C. H., Pattengale, P. K., and Ward, J. M., *Color Atlas of Hematopoietic Pathology of Mice*, Toxicology Pathology Associates, Little Rock, AK, 1985.
17. Foster, H. L., Small, J. D., and Fox, J. G., *The Mouse in Biomedical Research*, vols. I, II, III, and IV, Academic Press, New York, 1982, 1983.
18. Fox, J. G., Cohen, B. J., and Loew, F. M., Eds., *Laboratory Animal Medicine*, Academic Press, New York, 1984.
19. Glaister, J. R., *Principles of Toxicological Pathology*, Taylor & Francis Group, Philadelphia, PA, 1986.
20. Goodman, D. G. and Strandberg, J. D., Neoplasms of the female reproductive tract, in *The Mouse in Biomedical Research, IV. Experimental Biology and Oncology*, Foster, H. L., Small, J. D., Fox, J. G., Eds., Academic Press, New York, 1982, p. 397.
21. Haseman, J. K., Huff, J., and Boorman, G. A., Use of historical control data in carcinogenicity studies in rodents, *Toxicol. Pathol.*, 12, 126, 1984.

22. Hall, W. C., Ganaway, J. R., Rao, G. N., Peters, R. L., Allen, A. M., Luczak, J. W., Sandberg, E. M., and Quigley, B. H., Histopathologic observations in weanling B6C3F1 mice and F344/N rats and their adult parental strains, *Toxicol. Pathol.*, 20, 146, 1992.
23. Harkness, J. E. and Wagner, J. E., *The Biology and Medicine of Rabbits and Rodents*, Lea and Febiger, Philadelphia, PA, 1989.
24. Hollander, C. F., Delort, P., and Duprat, P., Toxicology in the older age group, in *Progress in Predictive Toxicology*, Clayson, D. B., Munro, I. C., Shubik, P., and Swenberg, J. A., Eds., Elsevier, New York, 1990, p. 113.
25. Jones, T. C., Mohr, U., and Hunt, R. D., Eds., *Monographs on Pathology of Laboratory Animals*, Springer-Verlag, New York, series sponsored by International Life Sciences Institute: *Endocrine System*, 1983; *Digestive System*, 1985; *Respiratory System*, 1985; *Urinary System*, 1986; *Genital System*, 1987; *Nervous System*, 1988; *Integument and Mammary Glands*, 1989; *Hematopoietic System*, 1990; *Cardiovascular and Musculoskeletal System*, 1991; *Eye and Ear*, 1991.
26. Maita, K., Hirano, M., Harada, T., Mitsumori, K., Yoshida, A., Takahashi, K., Nagashima, M., Kitazawa, T., Enomoto, A., Inui, K., and Shirasu, Y., Mortality, major cause of moribundity, and spontaneous tumors in CD1 mice, *Toxicol. Pathol.*, 16, 340, 1988.
27. Percy, D. H. and Barthold, S. W., *Pathology of Laboratory Rodents and Rabbits*, Iowa State University Press, Ames, IA, 1993.
28. Ribelin, W. E. and McCoy, J. R., *The Pathology of Laboratory Animals*, Charles C. Thomas, Springfield, IL, 1965.
29. Squire, R. A., Goodman, D. G., Valerio, M. G., Fredrickson, T., Strandberg, J. D., Levitt, M. H., Lingeman, C. H., Harshbarger, J. C., and Dawe, C. J., Tumors, in *Pathology of Laboratory Animals*, vol. II, Benirschke, K., Garner, F. M., and Jones, T. C., Eds., Springer-Verlag, New York, 1978, p. 1051.
30. Tamano, S., Hagiwara, A., Shibata, M., Karuta, Y., Fukushima, S., and Ito, N., Spontaneous tumors in aging (C57BL/6N × C3H/HeN) F1 (B6C3F1) mice, *Toxicol. Pathol.*, 16, 321, 1988.
31. Ward, J. M., Goodman, D. G., Squire, R. A., Chu, K. C., and Linhart, M. S., Neoplastic and nonneoplastic lesions in aging (C57BL/6N × C3H/HeN)F1 (B6C3F1) mice, *J. Natl Cancer Inst.*, 63, 849, 1979.
32. Haseman, J. K., Elwell, M. R., and Hailey, J. R., Neoplasm incidences in B6C3F₁ mice: NTP historical data, in *Pathology of the Laboratory Mouse*, Manonpot, R. R., Boorman, G. A., and Gaul, B., Eds., Cache River Press, Vienna, IL, 1999, pp. 679–689.
33. Baker, H. J., Lindsey, J. R., and Weisbroth, S. H., Eds., *The Laboratory Rat. I. Biology and Diseases*, Academic Press, New York, 1979.
34. Boorman, G. A., Eustis, S. L., Elwell, M. R., Montgomery, C. A. Jr., and MacKenzie, W. F., Eds., *Pathology of the Fischer Rat*, Academic Press, New York, 1990.
35. Burek, J. D., *Pathology of the Aging Rats*, CRC Press, Boca Raton, FL, 1978.
36. Coleman, G. L., Barthold, S. W., Osbaldiston, G. W., Foster, S. J., and Jonas, A. M., Pathological changes during aging in barrier-reared Fischer 344 male rats, *J. Gerontol.*, 32, 258, 1977.
37. Goodman, D. G., Ward, J. M., Squire, R. H., Chu, K. C., and Linhart, M. S., Neoplasms and nonneoplastic lesions in aging F344 rats, *Toxicol. Appl. Pharmacol.*, 48, 237, 1979.
38. Greaves, P. and Faccini, J. M., *Rat Histopathology*, Elsevier, New York, 1984.
39. Haseman, J. K., Huff, J. E., Rao, G. N., Arnold, J. E., Boorman, G. A., and McConnell, E. E., Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N × C3H/HeN)F1 (B6C3F1) mice, *J. Natl Cancer Inst.*, 75, 975, 1985.
40. Jacobs, B. B. and Huseby, R. A., Neoplasms occurring in aged Fischer rats with special reference to testicular, uterine, and thyroid tumors, *J. Natl Cancer Inst.*, 39, 303, 1967.
41. McMartin, D. N., Sahota, P. S., Gunson, D. E., Han Hsu, H., and Spaet, R. H., Neoplasms and related proliferative lesions in control Sprague-Dawley rats from carcinogenicity studies. Historical data and diagnostic considerations, *Toxicol. Pathol.*, 20, 212, 1992.
42. Mohr, U., Dungworth, D. L., and Capen, C. C., Eds., *Pathobiology of the Aging Rat*, vols. 1 and 2, International Life Sciences Institute, Washington, DC, 1992, 1993.
43. Sacksteder, M. R., Occurrence of spontaneous tumors in the germfree F334 rat, *J. Natl Cancer Inst.*, 57, 1371, 1976.
44. Sass, B., Rabstein, L. S., Madison, R., Nims, R. M., Peters, R. L., and Kelloff, G. J., Incidence of spontaneous neoplasms in F334 rats throughout the natural lifespan, *J. Natl Cancer Inst.*, 54, 1449, 1975.
45. Sher, S. P., Jensen, R. D., and Bokelman, D. L., Spontaneous tumors in control F334 and Charles River-CD rats and Charles River-CD1 and B6C3F1 mice, *Toxicol. Lett.*, 11, 103, 1982.
46. Solleveld, H. A., Haseman, J. K., and McConnell, E. E., Natural history of body weight gain, survival, and neoplasia in the F344 rat, *J. Natl Cancer Inst.*, 72, 929, 1984.
47. Stinson, S. F., Schuller, H. M., and Resznik, G., *Atlas of Tumor Pathology of the Fischer Rat*, CRC Press, Boca Raton, FL, 1989.
48. Fritz, T. E., Semen, R. C., Poole, C. M., and Norris, W. P., Studies on the spontaneous disease and pathology in the experimental beagle, *Argonne Nat. Lab. Biol. Med. Res. Div. Ann. Rep.*, ANL7278, 114, 1966.
49. Fritz, T. E., Semen, R. C., and Norris, W. P., Studies on the spontaneous disease and pathology in the experimental beagle colony, *Argonne Nat. Lab. Biol. Med. Res. Div. Ann. Rep.*, ANL7409, 283, 1967.
50. Hottendorf, G. H. and Hirth, R. S., Lesions of spontaneous subclinical disease in beagle dogs, *Vet. Pathol.*, 11, 240, 1974.
51. Maita, K., Masuda, H., and Suzuki, Y., Spontaneous lesions detected in the beagles used in toxicity studies, *Exp. Anim.*, 26, 161, 1977.
52. Oghiso, Y., Fukuda, S., and Hda, H., Histopathologic studies on distribution of spontaneous lesions and age changes in the beagle, *Jpn. J. Vet. Sci.*, 44, 941, 1982.
53. Thomassen, R. W., The dog: Pathology, in *Animal Models in Toxicology*, Gad, S. C. and Chengelis, Eds., C. P. Marcel Dekker, New York, 1992, pp. 600–674.
54. Pick, J. R. and Eubanks, J. W., A clinicopathologic study of heterogeneous and homogenous dog populations in North Carolina, *Lab. Anim. Care*, 15(1), 11, 1965.
55. Barron, C. N. and Saunders, L. Z., Visceral larva migrans in the dog, *Pathol. Vet.*, 3, 315, 1966.
56. Schiavo, D. M. and Field, W. E., The incidence of ocular defects in a closed colony of beagle dogs, *Lab. Anim. Sci.*, 24, 51, 1974.
57. Heywood, R., Hepworth, P. L., and Van Abbe, N. J., Age changes in the eyes of the beagle dog, *J. Small Anim. Pract.*, 17, 171, 1976.
58. Hirth, R. S. and Hottendorf, G. H., Lesions produced by a new lungworm in beagle dogs, *Vet. Pathol.*, 10, 385, 1973.
59. Kirchner, B. K., Port, C. D., Magoc, T. J., Sidor, M. A., and Ruben, Z., Spontaneous bronchopneumonia in laboratory dogs infected with untyped *Mycoplasma* spp., *Lab. Anim. Sci.*, 40, 625, 1990.

60. Giles, R. C., Kwapien, R. P., Geil, R. G., and Casey, H. W., Mammary nodules in beagle dogs administered investigational oral contraceptive steroids, *J. Natl Cancer Inst.*, 60(6), 1351, 1978.
61. Nelson, L. W., Weikel, J. H., Jr., and Reno, F. E., Mammary nodules in dogs during four years' treatment with megestrol acetate or chlormadinone acetate, *J. Natl Cancer Inst.*, 51(4), 1303, 1973.
62. Benjamin, S. A., Lee, A. C., and Saunders, W. J., Classification and behavior of canine mammary epithelial neoplasms based on lifespan observations in beagles, *Vet. Pathol.*, 36, 423, 1999.
63. Moulton, J. E., *Tumors in Domestic Animals*, University of California Press, Los Angeles, CA, 1990.
64. Hartman, H. A., Robinson, R. L., and Visscher, G. E., Naturally occurring intracytoplasmic eosinophilic inclusions in the canine exocrine pancreas, *Vet. Pathol.*, 12, 210, 1975.
65. Hargis, A. M., Integument system, in *Special Veterinary Pathology*, Thomson, R. G., Eds., Decker, Philadelphia, PA, 1988, pp. 1–68.
66. Muller, G. H., Scott, D. W., Miller, W. H., and Griffin C. E., *Muller and Kirk's Small Animal Dermatology*, 5th edition, Saunders, Philadelphia, PA, 1995.
67. Yager, J. A. and Scott, D. W., The skin and appendages, in *Pathology of Domestic Animals*, Jubb, K. V. F., Kennedy, P. C., and Palmer, N., Eds., Academic Press, San Diego, CA, 1985, pp. 407–549.
68. Fritz, T. E., Lombard, L. S., Tyler, S. A., and Norris, W. P., Pathology and familial incidence of orchitis and its relationship to thyroiditis in a closed beagle colony, *Exp. Mol. Pathol.*, 24, 142, 1976.
69. Cain, G. R., Tsai, K., Pulley, L. T., and Taylor, M., Detrusor myopathy in young beagle dogs, *Toxicol. Pathol.*, 28(4), 565, 2000.
70. Ringler, D. F. and Peter, G. K., Dogs and cats as laboratory animals, in *Laboratory Animal Medicine*, Fox, J. G., Cohen, B. J., and Loew, F. M., Eds., Academic Press, New York, 1984, pp. 241–271.
71. Aiello, S. E. and Moses, M. A., *The Merck Veterinary Manual*, 8th edition, Merck & Co., Whitehouse Station, NJ, 1998.
72. Bonagura, J. D. and Kirk, R. W., *Kirk's Current Veterinary Therapy XII: Small Animal Practice*, Saunders, Philadelphia, PA, 1995.
73. Bonagura, J. D., *Kirk's Current Veterinary Therapy XIII Small Animal Practice*, Saunders, Philadelphia, PA, 2000.
74. Carter, G. R., *Microbial Diseases: A Veterinarian's Guide to Laboratory Diagnosis*, 1st edition, Iowa State University Press, Ames, IA, 1993.
75. Ettinger, S. J. and Feldman, E. C., *Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat*, 4th edition, Saunders, Philadelphia, PA, 1995.
76. Jones, C. T., Hunt, R. D., and King, N. W., *Veterinary Pathology*, 6th edition, Williams & Wilkins, Baltimore, MD, 1997.
77. Jubb, K. V. F., Kennedy, P. C., and Palmer, N., *Pathology of Domestic Animals*, 4th edition, Academic Press, San Diego, CA, 1993.
78. Summer, B. A., Cummings, J. F., and DeLahunta, A., *Veterinary Neuropathology*, Mosby, St. Louis, MO, 1995.
79. Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M., and Jennings, F. W., *Veterinary Parasitology*, 2nd edition, Blackwell Science, Cambridge, MA, 1996.

16 Clinical Pathology

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BLOOD VOLUMES

The volume of blood in healthy mammals is relatively constant and typically represents about 5%–9% of body weight, depending on species.¹ Blood volumes of $\approx 1\%$ of body weight can be collected during the course of a study (i.e., ≈ 2.5 mL for a 250 g rat); however, this may cause minimal to moderate stress and/or result in a mild compensatory response by the bone marrow. For frequent periodic sampling, for example,

once weekly, blood volumes of ≈ 0.5 – 0.75 mL can be collected from a 250–300 g rat without any untoward effects. Somewhat larger volumes, for example, ≈ 1.5 – 2.0 mL, can be obtained routinely from this size animal on a monthly basis. At necropsy, about 50% of an animal's total blood volume can be obtained using suitable techniques as subsequently discussed. Approximate blood volumes for typical species used in toxicology research are shown in Table 16.1.

TABLE 16.1
Approximate Blood Volumes in Animals Typically Used
in Nonclinical Toxicology Research

Species	Typical Body Weight (kg)	Blood Volume (mL)			
		Total Volume (mL)	Weekly Sampling	Monthly Sampling	At Necropsy
Mouse	0.03	2	0.075	0.2	1
Rat	0.3	20	1	2	10
Dog	12.0	1000	50	100	400
Monkey ^a	3.0	200	10	20	100
Rabbit	3.0	200	10	20	100

Source: Modified from Loeb, W.F. and Quimby, F.W., *The Clinical Chemistry of Laboratory Animals*, Pergamon Press, New York, 1989; Levine, B.S., unpublished data, 1979–1993.

^a Rhesus or cynomolgus.

BLOOD SAMPLING TECHNIQUES

Numerous sampling techniques are available to collect blood samples in toxicology research. Table 16.2 contains those procedures that are used most typically. Terminal sample procedures listed are usually utilized only when a nonterminal sample procedure would not provide sufficient quantities of blood.

Collection of blood samples from the orbital sinus of rodents is well documented and has been described by several authors.^{2,3} The technique is rapid for experienced technicians; however, proficiency can be slow due to the nature of the procedure. One disadvantage is the inability to collect samples by this technique for use in coagulation studies.⁴ The orbital sinus procedure results in mild tissue damage releasing tissue thromboplastin, which results in prolonged prothrombin times (PTs).

Blood sampling from the jugular vein of rats has become popular and has been described by Meeks.⁵ The procedure can be performed with or without anesthesia and does not produce physical trauma, such as that encountered in the orbital sinus technique. It is quite rapid, and Meeks⁵ indicates that two experienced people can collect blood samples from rats at a rate of 1 every 30s. Blood samples can also be obtained from the tail vein of rodents by tail tip transection or tail vein venipuncture. The former procedure may result in somewhat diluted samples, that is, blood samples contaminated with tissue fluids. Although cardiac puncture has been successfully used to collect blood samples in some laboratories, the associated risks, including blood leakage into the pericardial sac, tend to outweigh any potential advantage over other, more accepted procedures.

Blood collection procedures in canines are typically limited to the cephalic and jugular veins. Although the jugular vein technique may require more training (by technicians and dogs) than sampling from the cephalic vein, the ability to sample repetitively from the jugular vein of dogs

suggests that this procedure is more advantageous. This is especially critical in pharmacokinetic studies in which rapid and very repetitive sampling is required. In addition, repetitive sampling from the cephalic vein of dogs, especially by inexperienced technicians, often results in localized tissue damage. Alternatively, a cephalic vein catheter, protected from self-withdrawal, can be used.

For the measurement of routine clinical chemistry tests, venous blood samples typically are collected. If blood gases are to be measured, for example, PO₂, PCO₂, bicarbonate concentration, and pH, arterial samples are preferred; however, venous samples will provide reliable data.⁶

TABLE 16.2
Blood Sampling Techniques Used
in Nonclinical Toxicology Research

Species	Technique
Mouse	Orbital sinus
	Cardiac puncture
Rat	Orbital sinus
	Jugular vein
	Cardiac puncture
	Tail vein
	Aorta (terminal sample)
Dog	Vena cava (terminal sample)
	Jugular vein
	Cephalic vein
Monkey ^a	Femoral vein/artery
	Cephalic vein
Rabbit	Jugular vein
	Marginal ear vein
	Cardiac puncture

^a Rhesus or cynomolgus.

BLOOD SAMPLE REQUIREMENTS FOR CLINICAL PATHOLOGY TESTS

Most current clinical chemistry and hematology instruments require relatively small samples for analysis. In hematology testing, a 20–50 μL sample (depending on the instrument) obtained with a suitable anticoagulant such as the potassium salt of EDTA is sufficient to measure directly the following parameters: white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, mean corpuscular volume (MCV), and platelet count. From these measurements, the following variables can be calculated: hematocrit (Hct) (packed cell volume), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Many modern instruments can also directly measure mean platelet volume (MPV).

In addition to the previously described hematology tests, a WBC differential and a reticulocyte count each requires about 50 μL , when assessed from separate blood smears with appropriate stains. A few instruments recently adopted for animal clinical pathology studies can measure these parameters directly; however, their cost, in addition to other factors, has not resulted in their routine use. Thus, a hematology sample of ≈ 300 –400 μL collected in Microtainer® tubes coated with K^+ -EDTA is typically sufficient to measure routine hematologic variables necessary for nonclinical toxicity evaluations.

Additional hematology assays, which may be required in selected nonclinical toxicology studies, include methemoglobin, carboxyhemoglobin, fibrinogen, prothrombin (PT), and activated partial thromboplastin time (APTT). The first two can be measured with a co-oximeter and can utilize the K^+ -EDTA sample mentioned previously. Since methemoglobin, the oxidized form of Hb, is somewhat unstable because the blood enzyme methemoglobin reductase will reduce it back to Hb, these samples should be collected on ice and assayed within 1 h. PT and APTT should be measured from blood samples collected with citrate as the anticoagulant. Because the ratio of the citrate anticoagulant to blood sample is critical, exactly 1 mL of citrated blood must be obtained for coagulation tests using a tuberculin syringe prefilled with 0.1 mL of citrate solution. Larger samples can be collected in Vacutainer® tubes that automatically fill to a predetermined volume, for example, 2.0 mL (0.2 mL liquid sodium citrate and 1.8 mL blood). Methemoglobin analysis by co-oximetry typically requires less than 100 μL .

When modern instrumentation is used, the volume of serum/plasma required for routine clinical chemistry assays is relatively small, for example, 5–30 μL per test. Thus, the measurement of approximately 15–20 clinical chemistry parameters usually requires about 400–500 μL of serum/plasma. This, in turn, typically requires about 1.0–1.2 mL whole blood. To account for possible repeat analysis, poor serum yield, etc., approximately 1.5–1.8 mL of blood is routinely collected for a typical battery of hematology and clinical chemistry tests exclusive of coagulation assays (i.e., PT, APTT, and fibrinogen) and methemoglobin analysis.

CLINICAL CHEMISTRY PARAMETERS

The development of clinical chemistry tests as diagnostic tools in nonclinical toxicology research is based on their use in disease diagnosis in clinical medicine. Thus, many of the routine tests are virtually identical with measurements performed in human clinical laboratories. Fortunately, many of the diagnostic tests adapted from human medicine are appropriate for identifying organ system damage in animals exposed to toxic doses of drugs and other chemicals. Certain clinical chemistry tests, however, are not appropriate, for example, uric acid levels in laboratory animals. This analyte, typically used to assess renal function in humans, is rapidly converted to allantoin in animals (except for Dalmatian dogs), which is not suitable for routine assay. It should be noted that commercial clinical chemistry reagents currently used in animal testing are for assay of human analytes. Thus, optical conditions for measurement of laboratory animal analytes may not be met by these reagents. The reader should consult other textbooks for an in-depth discussion on the significance of altered clinical chemistry measurements with regard to the diagnosis of disease, including those that are chemically induced.^{7,8}

In 1992, a joint task force of the American Association for Clinical Chemistry's Division of Animal Clinical Chemistry (AACC-DACC) and the American Society for Veterinary Clinical Pathology (ASVCP) published clinical pathology testing recommendations in animals used in nonclinical toxicity testing programs.⁹ These expert recommendations provided a standardized approach to the harmonization of clinical pathology testing in laboratory animals used in nonclinical toxicology research. The paper suggests general and specific approaches, and typical clinical chemistry tests that fulfill these testing requirements are summarized in Table 16.3.

The primary function of serum/plasma clinical chemistry testing in nonclinical safety assessment testing is to determine potential target organs of toxicity and the associated time course of that damage without the need of biopsy or necropsy samples. Histopathology studies typically performed at the end of a study support clinical pathology findings. In certain instances, the measurement of increased levels of certain analytes, for example, enzymes, does not always indicate the specific target organ that is affected. Several organs contain reasonably high levels of the same enzyme but in different isozyme forms. Thus, without suitable isozyme analysis, the assignment of specific organs at risk is sometimes fraught with uncertainty. Examples include lactate dehydrogenase (LDH; liver, muscle, heart), aspartate aminotransferase (AST) (liver, muscle, RBCs), and creatine phosphokinase (liver, muscle).

Serum corticosteroid levels are not typically measured in safety assessment studies but can be useful when drug-induced stress occurs or a debilitating condition is seen. Effects secondary to elevated corticosteroid levels include leukocytosis and thymic lymphocyte depletion.

TABLE 16.3
Routine Clinical Chemistry Testing
Recommendations in Nonclinical Safety
Assessment Studies

Core Battery	
Albumin	Glucose
Calcium	Phosphorus, inorganic
Chloride	Protein, total
Creatinine	Potassium
Cholesterol, total	Sodium
Globulin	Urea nitrogen (BUN)
Hepatocellular Health	Hepatobiliary Health
(Minimum of two tests)	(Minimum of two tests)
Alanine aminotransferase (ALT)	Alkaline phosphatase
Aspartate aminotransferase (AST)	Gamma glutamyltransferase (γGT)
Sorbitol dehydrogenase (SDH)	5'-nucleotidase
Total bile acids	Total bile acids

Source: Modified from Weingand, K. et al., *Toxicol. Pathol.*, 20, 539, 1992.

HEMATOLOGY PARAMETERS

As described previously for clinical chemistry tests, hematology measurements in nonclinical toxicology research are also based on human diagnostic disease procedures. Hematology assays give vital information regarding the status of bone marrow activity, as well as potential intravascular effects, for example, hemolysis. Hematology measurements and their significance in diagnostic veterinary medicine and nonclinical toxicology research are detailed in several textbooks that are beyond the scope of this chapter.^{10,11}

The advent of hematology instrumentation has resulted in significantly greater accuracy and precision over manual methods. Many hematology instruments currently used in animal research can be adjusted for species differences in erythrocyte size. This allows for the determination of an accurate RBC count and subsequent calculations of packed cell volume (Hct), MCH, and MCHC. Although WBC differential counts are routinely determined in the laboratory as a percent of total WBCs, they must be multiplied by the total WBC count to result in quantitatively meaningful data, that is, absolute differential WBC counts.⁹ Leukocytes are classified by function into lymphocytes, neutrophils, eosinophils, basophils, and monocytes. Lymphocytes and neutrophils predominate, with one cell type typically seen in greater numbers, depending on the species as follows: lymphocytes (rats, mice, rabbits), neutrophils (dogs), and mixed (monkeys).

The AACC-DACC/ASVCP Joint Task Force publication described previously also includes hematology recommendations in nonclinical toxicology testing.⁹ Those recommendations are shown in Table 16.4. However, other tests may be appropriate depending on the type of agent under investigation. These include but are not limited to the following: methemoglobin levels, RBCs with Heinz bodies, RBCs with Howell–Jolly bodies, and fibrinogen levels.

TABLE 16.4
Routine Hematology Testing Recommendations
in Nonclinical Safety Assessment Studies

Parameters	
APTT	MCH
Erythrocyte count	MCHC
Hct	MCV
Hb	Platelet count
Leukocyte count, total	PT
Leukocyte count, differential	Reticulocyte count ^a

Source: Adapted from Weingand, K. et al., *Toxicol. Pathol.*, 20, 539, 1992.
^a Slides should be prepared, but only evaluated if signs of anemia are present.

URINALYSIS PARAMETERS

Urinalysis measurements are primarily concerned with renal function and can aid in the identification of chemically induced nephrotoxicity. The method of urine sample collection can alter the urinalysis, and a fresh sample may be preferred if urine volume is not necessary. This can be obtained directly from the bladder at necropsy or by physically manipulating the animal. If a timed (e.g., 16–24 h) urine volume is necessary, it may be useful to collect the sample into a cooled vessel or into one that contains a preservative.

The previously described AACC-DACC/ASVCP Joint Task Force publication includes recommendations for the conduct of urinalysis in safety assessment studies, which are shown in Table 16.5.⁹ Urinalysis measurements are typically of limited usefulness and may only be appropriate if kidney toxicity is suspected from preliminary studies. Refer to Chapter 9 for detailed information on renal function parameters for various rat strains and Table B.13 in Appendix B for other species.

TABLE 16.5
Routine Urinalysis Testing Recommendations
in Nonclinical Safety Assessment Studies

Recommended	
Specific gravity	
Volume (timed, 16–24 h)	
Optional	
Semiquantitative (reagent strips), for example, microscopic examination of spun sediment	Bilirubin, glucose, ketones, occult blood, pH, protein, urobilinogen

Source: Adapted from Weingand, K. et al., *Toxicol. Pathol.*, 20, 539, 1992.

REFERENCE VALUES

Although concurrent control animals should always be included in any aspect of toxicology research, the availability of in-house historical control clinical pathology data is often useful in data interpretation. Laboratories actively engaged in nonclinical toxicology research should therefore establish in-house reference ranges during the early phase of their testing programs. Although helpful for comparative purposes, control animal data from the literature as well as from other laboratories are inappropriate for use as in-house historical control databases. Clinical pathology test values are affected by numerous variables, as summarized in Table 16.6.

In an attempt to present “typical” control animal clinical pathology ranges, literature data in addition to unpublished observations have been used. The ranges presented are approximate and are shown for mean values, not for individual data points. Thus, estimates of variability within a given set of experimental measurements are not presented. These data sets were obtained under many different experimental conditions, for example, source of animals, diet, fasted or nonfasted,

caging conditions, environmental conditions, blood collection site, anesthetic, and instrumentation. The reader is directed to consult the references provided for specific details.

As mentioned previously, urinalysis measurements are usually of limited usefulness, unless kidney toxicity is evident. In general, semiquantitative measurements using reagent strips as shown in Table 16.5 typically yield negative to trace results in all species, whereas microscopic examination of a spun sediment yields negative to occasional quantities of cells, casts, crystals, bacteria, etc. In laboratory animals, urinary specific gravity usually ranges from ≈ 1.000 to ≈ 1.060 , whereas pH typically ranges from 6 to 9.

Reference value data are presented in Tables 16.7 through 16.13 (clinical chemistry) and Tables 16.14 through 16.20 (hematology). Tables have been arranged by species, strain, and age. Generally, sex effects are either minimal or not apparent in laboratory animal clinical pathology measurements. Where significant differences do exist, for example, serum alkaline phosphatase, cholesterol, protein and triglyceride levels, and leukocyte counts, both ranges are shown.

TABLE 16.6
Factors That Can Affect Clinical Chemistry and Hematology Test Measurements

Biologic	Methodologic
Species	Blood collection site
Sex	Anesthetic use
Age	Instrumentation
Fasting conditions	Assay conditions, that is, temperature,
Diet	substrate concentration, etc.

TABLE 16.7
Mean Control Ranges of Typical Serum Clinical Chemistry Measurements in CD® Rats

Parameter	10–12 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	108–110 Weeks Old
ALT (IU/L)	10–40	10–50	10–50	20–60	20–60	20–60
Albumin (g/dL)	3.4–4.1 (M) 3.5–4.5 (F)	3.3–4.2 (M) 3.5–4.7 (F)	3.5–4.0 (M) 4.0–5.0 (F)	3.0–3.8 (M) 3.5–4.5 (F)	3.0–4.0 (M) 3.7–4.5 (F)	2.7–3.5 (M) 3.3–3.7 (F)
Albumin/globulin ratio	1.0–1.5	1.0–1.5	1.0–1.5	0.75–1.75	0.75–1.75	0.75–1.5
Alkaline phosphatase (IU/L)	140–300 (M) 80–100 (F)	50–150 (M) 25–150 (F)	50–150 (M) 25–100 (F)	50–150 (M) 25–100 (F)	50–150 (M) 25–100 (F)	50–100 (M) 25–100 (F)
AST (IU/L)	45–90	45–100	45–120	60–120	75–150	75–150
Bile acids, total ($\mu\text{mol/L}$)	20–60	20–60	—	—	—	—
Bilirubin, total (mg/dL)	0.2–0.4	0.1–0.5	0.1–0.5	0.1–0.5	0.1–0.5	0.1–0.4
Calcium (mg/dL)	9.8–12.0	9.8–12.0	9.8–12.0	9.8–12.0	9.8–12.0	9.8–12.0
Chloride (mEq/L)	97–105	97–105	95–105	97–105	97–105	95–105
Cholesterol, total (mg/dL)	50–85	50–100	70–140	60–150	130–180 (M) 100–150 (F)	130–180 (M) 90–150 (F)
Creatine kinase (CK) (IU/L)	50–400	50–300	50–500	—	—	—
Creatinine (mg/dL)	0.3–0.8	0.3–0.9	0.3–1.0	0.4–0.8	0.4–0.8	0.4–1.3
γGT (IU/L)	0–2	0–2	0–3	0–5	0–7	0–5

(continued)

TABLE 16.7 (continued)**Mean Control Ranges of Typical Serum Clinical Chemistry Measurements in CD® Rats**

Parameter	10–12 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	108–110 Weeks Old
Globulin (g/dL)	2.5–4.0	2.5–4.0	2.0–4.5	2.0–4.5	2.0–4.5	2.0–4.5
Glucose (mg/dL)	90–175	100–175	100–200	100–200	100–175	100–175
LDH (IU/L)	50–400	50–400	50–500	—	—	—
Phosphorus, inorganic (mg/dL)	7.0–10.0	4.0–8.5	4.0–8.0	3.5–7.0	3.5–8.0	4.0–7.0
Potassium (mEq/L)	5.5–8.0	4.0–7.0	4.0–7.0	4.0–7.0	3.5–6.0	3.5–6.0
Protein, total (g/dL)	6.2–7.6 (M) 6.3–8.2 (F)	6.2–7.8 (M) 6.5–8.5 (F)	6.2–8.0 (M) 7.0–9.0 (F)	6.0–8.0 (M) 6.5–8.5 (F)	6.3–7.6 (M) 6.7–8.0 (F)	5.7–6.5 (M) 6.3–7.1 (F)
Sodium (mEq/L)	140–153	140–153	140–153	140–153	140–153	140–145
SDH (IU/L)	10–30	10–30	10–30	—	—	—
Triglycerides (mg/dL)	50–125	50–200	50–200	50–300	75–400	50–300
BUN (mg/dL)	12–18	12–20	12–20	12–18	12–18	12–30

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Charles River Laboratories, Serum chemistry parameters for the CrI:CD®BR rat, Wilmington, MA, 1993.

Note: —, data unavailable.

TABLE 16.8**Mean Control Ranges of Typical Serum Clinical Chemistry Measurements in F-344 Rats**

Parameter	12–14 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	110–112 Weeks Old
ALT (IU/L)	25–45	30–62	20–40	56–100 (M) 33–65 (F)	41–80 (M) 32–50 (F)	25–60
Albumin (g/dL)	3.8–4.7	3.0–5.0	4.0–5.0	3.8–5.0	3.8–5.0	3.5–5.0
Albumin/globulin ratio	1.5–2.3	1.1–2.5	1.5–2.0	1.4–1.9	1.4–2.0	1.2–1.8
Alkaline phosphatase (IU/L)	200–300 (M) 150–250 (F)	58–154 (M) 45–120 (F)	45–80	31–68	—	—
AST (IU/L)	50–90	50–100	—	—	—	—
Bile acids, total (μmol/L)	10–50	—	—	—	—	—
Bilirubin, total (mg/dL)	—	0.1–0.5	0.1–0.4	0.1–0.5	0.1–0.5	0.1–0.4
Calcium (mg/dL)	—	9.5–12.0	9.5–11.2	9.5–11.5	9.5–11.5	9.8–11.7
Chloride (mEq/L)	—	97–115	98–110	100–112	97–100	104–113
Cholesterol, total (mg/dL)	70–110 (M) 90–135 (F)	50–80 (M) 80–120 (F)	50–80 (M) 85–130 (F)	68–125 (M) 110–150 (F)	100–120	125–175
CK (IU/L)	60–300	100–400	300–700	300–500	100–500	100–400
Creatinine (mg/dL)	0.5–1.0	0.4–0.8	—	—	—	—
Globulin (g/dL)	1.5–2.5	1.2–2.8	2.0–3.0	2.3–3.5	2.0–3.0	2.2–3.2
Glucose (mg/dL)	100–180	90–170	80–130	90–140	90–140	90–140
LDH (IU/L)	—	500–800	—	—	—	—
Phosphorus, inorganic (mg/dL)	—	3.9–7.3	400–800	150–400	—	—
Potassium (mEq/L)	—	3.6–5.9	4.0–5.7	4.1–5.5	4.0–5.2	4.0–5.1
Protein, total (g/dL)	6.0–7.2	5.7–7.6	6.2–7.5	6.5–7.6	6.0–7.8	6.1–8.0
Sodium (mEq/L)	—	140–155	142–158	142–156	138–149	138–146
SDH (IU/L)	15–60	5–25	5–35	—	—	—
Triglycerides (mg/dL)	100–400 (M) 25–130 (F)	75–150 (M) 35–70 (F)	125–190 (M) 30–70 (F)	90–175 (M) 40–85 (F)	110–240 (M) 60–145 (F)	80–220
BUN (mg/dL)	15–25	10–26	12–24	10–20	10–20	12–25

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; NIEHS, A summary of control values for F344 rats and B6C3F1 mice in 13 week subchronic studies, Program Resources, Research Triangle Park, NC, 1985; Burns, K.F. et al., *Lab. Anim. Sci.*, 21, 415, 1971.

Note: —, data unavailable.

TABLE 16.9**Mean Control Ranges of Typical Serum Clinical Chemistry Measurements in B6C3F₁ Mice**

Parameter	12–14 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	110–112 Weeks Old
ALT (IU/L)	20–50	25–100	22–90	20–50	23–60	23–60
Albumin (g/dL)	2.3–4.4	2.5–4.2	2.7–3.8	3.0–4.0	3.0–3.9	3.0–4.1
Albumin/globulin ratio	1.0–2.0	0.8–2.0	1.2–1.9	1.3–1.9	1.3–2.0	1.3–2.0
Alkaline phosphatase (IU/L)	30–80 (M) 40–140 (F)	20–55 (M) 45–85 (F)	—	—	—	—
AST (IU/L)	40–110	64–180	—	—	—	—
Bilirubin, total (mg/dL)	—	0.1–0.5	0.1–0.5	0.1–0.5	0.1–0.5	0.1–0.5
Calcium (mg/dL)	—	8.2–11.8	—	—	—	—
Chloride (mEq/L)	—	110–128	—	—	—	—
Cholesterol, total (mg/dL)	90–160	80–130	85–150	80–150	90–160	90–175
CK (IU/L)	50–300	—	—	—	—	—
Creatinine (mg/dL)	0.3–0.8	0.2–0.8	—	—	—	—
Globulin (g/dL)	1.5–2.5	1.0–2.7	1.6–2.4	1.8–3.1	1.6–3.0	1.8–3.0
Glucose (mg/dL)	125–200	81–165	115–170	115–170	115–170	115–170
Phosphorus, inorganic (mg/dL)	—	—	—	—	—	—
Potassium (mEq/L)	—	3.6–7.3	—	—	—	—
Protein, total (g/dL)	4.5–5.5	4.0–6.0	4.2–6.2	4.8–6.5	4.8–6.6	5.4–6.5
Sodium (mEq/L)	—	147–163	—	—	—	—
SDH (IU/L)	15–50	18–57	—	—	—	—
Triglycerides (mg/dL)	75–175	75–130	100–173	90–190	110–160	90–175
BUN (mg/dL)	15–35	12–34	12–27	12–24	10–24	15–28

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; NIEHS, A summary of control values for F344 rats and B6C3F₁ mice in 13 week subchronic studies, Program Resources, Research Triangle Park, NC, 1985.

Note: —, data unavailable.

TABLE 16.10
Mean Control Ranges of Typical Serum Clinical Chemistry Measurements in
CD-1 and BALB/c Mice

Parameter	<1-Year-Old CD-1	>1-Year-Old CD-1	1–3-Month-Old BALB/c	6–12-Month-Old BALB/c
ALT (IU/L)	30–250 (M) 30–100 (F)	20–200 (M) 20–80 (F)	— —	— —
Albumin (g/dL)	—	—	1.6–2.6	1.3–2.6
Albumin/globulin ratio	—	—	—	—
Alkaline phosphatase (IU/L)	30–70	20–75	75–275	47–102
AST (IU/L)	75–300	75–300	40–140	70–110
Bilirubin, total (mg/dL)	0.2–0.8	0.2–0.8	0.5–1.2	0.4–1.0
Calcium (mg/dL)	8.5–11.5	6.7–11.5	7.8–10.8	6.5–9.6
Chloride (mEq/L)	110–125	110–135	—	—
Cholesterol, total (mg/dL)	90–170 (M) 60–125 (F)	60–170 (M) 50–100 (F)	165–295	100–300
CK (IU/L)	—	—	—	—
Creatinine (mg/dL)	0.3–1.0	0.2–2.0	—	—
Globulin (g/dL)	—	—	—	—
Glucose (mg/dL)	75–175	60–150	75–150	40–160
Phosphorus, inorganic (mg/dL)	7.5–11.0	6.0–10.0	4.5–8.9	4.7–7.2
Potassium (mEq/L)	6.5–9.0	6.6–9.0	—	—
Protein, total (g/dL)	4.5–6.0	3.5–5.6	4.4–6.0	4.4–6.4
Sodium (mEq/L)	145–160	155–170	—	—
Triglycerides (mg/dL)	60–140 (M) 50–100 (F)	40–150 (M) 25–75 (F)	— —	— —
BUN (mg/dL)	20–40	20–70	10–30	10–30

Sources: Modified from Frith, C.H. et al., *Lab. Anim. Sci.*, 30, 835, 1980; Wolford, S.T. et al., *Toxicol. Environ. Health*, 18, 161, 1986.

Note: —, data unavailable.

TABLE 16.11
Mean Control Ranges of Typical Serum Clinical Chemistry Measurements
in Beagle Dogs

Parameter	6–8 Months Old	9–11 Months Old	12–14 Months Old	15–18 Months Old	19–30 Months Old
ALT (IU/L)	20–40	20–40	20–40	20–40	20–40
Albumin (g/dL)	2.5–3.5	2.5–3.5	2.5–3.5	2.5–4.0	2.7–4.5
Albumin/globulin ratio	0.8–1.5	0.8–1.5	0.8–1.5	0.8–2.0	0.8–2.0
Alkaline phosphatase (IU/L)	120–160 (M) 100–130 (F)	70–120 (M) 60–100 (F)	50–100	35–100	35–100
AST (IU/L)	30–45	30–50	25–50	25–50	25–50
Bilirubin, total (mg/dL)	0.1–0.7	0.1–0.7	0.1–0.7	0.1–0.3	0.1–0.3
Calcium (mg/dL)	9.0–11.5	9.0–11.5	9.0–11.5	10.0–11.3	10.0–11.5
Chloride (mEq/L)	100–115	100–115	100–115	105–119	105–115
Cholesterol, total (mg/dL)	150–250	125–250	125–250	125–250	125–225
CK (IU/L)	100–400	100–400	100–400	—	—
Creatinine (mg/dL)	0.5–0.8	0.7–0.9	0.7–0.9	—	—
γGT (IU/L)	0–5	0–5	0–5	—	—
Globulin (g/dL)	2.5–3.5	2.5–3.5	2.5–3.5	2.5–3.5	2.5–3.5
Glucose (mg/dL)	100–130	100–130	100–130	70–110	70–110
Haptoglobin (mg/dL)	50–200	50–150	25–100	—	—
LDH (IU/L)	30–100	30–100	30–100	—	—
Phosphorus, inorganic (mg/dL)	6.0–9.0	4.0–6.0	3.0–5.0	3.0–5.0	3.0–4.7
Potassium (mEq/L)	4.2–5.0	4.2–5.0	4.2–5.0	4.1–5.1	4.2–5.2
Protein, total (g/dL)	5.5–6.5	5.5–6.5	5.5–6.5	5.5–6.5	5.7–6.6
Sodium (mEq/L)	143–147	143–147	143–147	143–153	143–153
Triglycerides (mg/dL)	30–60	30–75	30–75	—	—
BUN (mg/dL)	10–20	10–20	10–20	10–20	10–20

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Clarke, D. et al., *Am. Assoc. Clin. Chem.*, Special Issue, 17, 1992; Pickrell, J.A. et al., *Am. J. Vet. Res.*, 35, 897, 1974; Kaspar, L.V. and Norris, W.P., *Lab. Anim. Sci.*, 27, 980, 1977.

Note: —, data unavailable.

TABLE 16.12

Mean Control Ranges of Typical Serum Clinical Chemistry Measurements in Nonhuman Primates

Parameter	3–7-Year-Old Cynomolgus	1–2-Year-Old Rhesus	3–7-Year-Old Rhesus	<1.5-Year-Old Marmoset	>1.5-Year-Old Marmoset	1–5-Year-Old Baboon	6–15-Year-Old Marmoset
ALT (IU/L)	20–60	20–50	15–40	45–75	40–70	15–50	20–50
Albumin (g/dL)	3.5–4.8	3.0–4.5	3.2–4.5	3.5–5.8	3.5–5.8	3.1–4.5	2.0–4.5
Albumin/globulin ratio	1.0–1.5	1.0–1.5	1.0–1.5	1.0–1.5	1.0–1.5	1.0–1.5	1.0–1.5
Alkaline phosphatase (IU/L)	300–800 (M) 200–500 (F)	200–600	70–300	100–250	35–80	200–1000	100–200
Amylase (IU/L)	200–500	—	—	1000–2000	500–1500	200–400	200–500
AST (IU/L)	25–60	25–60	15–70	100–200	100–200	18–35	20–35
Bilirubin, total (mg/dL)	0.3–0.8	0.1–0.8	0.1–0.6	0.1–0.9	0.1–0.9	0.3–0.7	0.3–0.5
Calcium (mg/dL)	9.0–11.0	8.2–10.5	8.5–10.3	8.1–12.4	8.5–11.7	8.0–9.5	7.5–10.0
Chloride (mEq/L)	100–115	103–115	97–110	80–110	93–119	105–115	100–110
Cholesterol, total (mg/dL)	90–160	90–160	90–170	90–210	105–230	75–200	70–125
CK (IU/L)	140–200	200–1000	200–600	—	—	—	—
Creatinine (mg/dL)	0.7–1.2	0.5–0.9	0.7–1.2	0.2–1.0	0.2–1.0	0.8–1.2	1.0–1.8
γGT (IU/L)	40–90	—	10–60	—	—	—	—
Globulin (g/dL)	3.0–4.5	3.0–4.0	3.0–4.0	2.5–4.0	3.5–4.0	2.5–4.0	2.5–4.5
Glucose (mg/dL)	50–100	50–100	41–80	180–275	130–240	50–125	50–140
LDH (IU/L)	300–600	130–600	125–600	125–500	100–350	100–400	100–350
Phosphorus, inorganic (mg/dL)	4.0–7.0	3.2–5.0	3.0–5.3	5.5–9.8	4.0–7.5	4.7–7.5	1.3–4.5
Potassium (mEq/L)	3.0–4.5	3.0–4.2	3.1–4.1	3.5–5.0	3.0–4.8	3.2–4.3	3.7–4.8
Protein, total (g/dL)	7.0–9.0	6.7–8.0	7.0–8.3	5.5–7.5	6.0–8.0	6.0–8.0	6.0–7.5
Sodium (mEq/L)	140–153	144–150	142–148	150–170	150–170	142–158	142–158
Triglycerides (mg/dL)	30–70	50–200	50–200	75–200	75–200	25–60	30–125
BUN (mg/dL)	15–25	14–26	14–25	17–35	15–32	10–25	10–25

Sources: Modified from Clarke, D. et al., *Am. Assoc. Clin. Chem.*, Special Issue, 17, 1992; Levine, B.S., unpublished data, 1979–1993; Kapeghian, L.C. and Verlangieri, A.J., *J. Med. Primatol.*, 13, 97, 1984; Davy, C.W. et al., *Lab. Anim.*, 18, 135, 1984; Yarbrough, L.W. et al., *Lab. Anim. Sci.*, 34, 276, 1984; Hack, C.A. and Gleiser, C.A., *Lab. Anim. Sci.*, 32, 502, 1982.

Note: —, data unavailable.

TABLE 16.13
Mean Control Ranges of Typical Serum Clinical Chemistry Measurements
in New Zealand White Rabbits

Parameter	15–20 Weeks Old	25–40 Weeks Old	1–2 Years Old
ALT (IU/L)	25–70	25–70	25–70
Albumin (g/dL)	3.8–5.0	3.5–4.7	3.0–4.5
Albumin/globulin ratio	2.0–3.0	2.0–3.0	2.0–3.0
Alkaline phosphatase (IU/L)	50–120	40–120	15–90
AST (IU/L)	20–50	10–35	10–30
Bilirubin, total (mg/dL)	0.1–0.5	0.1–0.5	0.2–0.6
Calcium (mg/dL)	12.0–14.0	11.0–14.0	12.0–15.0
Chloride (mEq/L)	97–110	96–108	100–110
Cholesterol, total (mg/dL)	20–60	20–60	20–60
CK (IU/L)	200–800	200–1000	200–1000
Creatinine (mg/dL)	1.0–1.6	0.8–1.6	0.8–1.7
γ GT (IU/L)	—	0–10	0–6
Globulin (g/dL)	1.4–1.9	1.5–2.2	1.5–2.5
Glucose (mg/dL)	100–160	100–175	80–140
LDH (IU/L)	50–200	50–200	35–125
Phosphorus, inorganic (mg/dL)	4.6–7.2	4.0–7.0	3.0–5.0
Potassium (mEq/L)	4.0–5.2	4.0–5.0	3.3–4.5
Protein, total (g/dL)	5.4–6.6	5.5–7.0	5.5–7.5
Sodium (mEq/L)	132–144	132–145	132–150
BUN (mg/dL)	10–20	12–22	12–25

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Hewett, C.D. et al., *Clin. Chem.*, 35, 1777, 1989; Yu, L. et al., *Clin. Biochem.*, 12, 83, 1979.

Note: —, data unavailable.

TABLE 16.14
Mean Control Ranges of Typical Hematology Measurements in CD® Rats

Parameters	10–12 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	108–110 Weeks Old
APTT (s)	14.0–20.0 (M) 12.0–18.0 (F)	14.0–20.0 (M) 13.0–18.0 (F)	14.0–17.0 (M) 13.0–16.0 (F)	16.0–19.0 (M) 15.0–18.0 (F)	—	—
Erythrocyte count ($10^6/\text{mm}^3$)	6.8–8.5 (M) 7.0–8.2 (F)	7.0–9.8 (M) 6.5–9.2 (F)	7.0–9.6 (M) 6.5–8.8 (F)	7.0–9.2 (M) 6.5–8.5 (F)	7.0–9.2 (M) 6.0–8.5 (F)	6.2–8.2 (M) 5.8–8.0 (F)
Fibrinogen (mg/dL)	—	200–300 (M) 130–190 (F)	—	—	—	—
Hct (%)	40.0–48.0	36.0–52.0	36.0–50.0	38.0–48.0	38.0–50.0	35.0–45.0
Hb (g/dL)	14.0–17.0	14.0–17.0	14.0–17.0	14.0–17.0	14.0–17.0	12.0–15.0
Leukocyte count, total ($10^3/\text{mm}^3$)	6.0–18.0 (M) 4.0–14.0 (F)	6.0–19.0 (M) 5.0–14.0 (F)	6.0–18.0 (M) 4.0–11.0 (F)	5.0–15.0 (M) 3.0–9.0 (F)	10.0–15.0 (M) 6.0–10.0 (F)	5.0–18.0 (M) 3.0–12.0 (F)
MCH (pg)	19.0–22.0	16.0–20.0	17.0–21.0	16.0–21.0	16.0–20.0	16.0–20.0
MCHC (g/dL)	33.0–38.0	31.0–38.0	31.0–38.0	32.0–38.0	31.0–36.0	31.0–36.0
MCV (fl)	53.0–63.0	50.0–60.0	45.0–60.0	46.0–58.0	48.0–56.0	50.0–63.0
Methemoglobin (% Hb)	0.4–1.2	0.4–1.2	0.4–1.2	—	—	—
Platelet count ($10^3/\text{mm}^3$)	900–1300	800–1200	700–1200	700–1200	700–1200	700–1200
PT (s)	9.0–14.0	9.0–14.0	10.0–14.0	10.0–14.0	—	—
Reticulocyte count (% RBC)	0.2–1.0	0.2–0.8	0.2–0.8	—	—	—

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Charles River Laboratories, Hematology parameters for the Crl:CD®BR rat, Wilmington, MA, 1993.

Note: —, data not available.

TABLE 16.15
Mean Control Ranges of Typical Hematology Measurements in F-344 Rats

Parameters	10–12 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	108–110 Weeks Old
Erythrocyte count ($10^6/\text{mm}^3$)	7.2–8.6	7.0–10.0	8.5–9.6	7.2–9.5	7.5–9.8	6.5–9.6
Hct (%)	39.5–45.5	42.0–50.0	41.4–46.7	40.0–46.6	40.3–45.5	40.0–48.5
Hb (g/dL)	15.0–17.0	15.0–17.3	15.0–17.8	15.7–17.5	15.5–17.6	13.0–18.5
Leukocyte count, total ($10^3/\text{mm}^3$)	7.1–13.5 (M) 5.4–11.7 (F)	6.5–10.7 (M) 4.5–7.0 (F)	6.5–8.7 (M) 4.4–6.5 (F)	5.8–9.0 (M) 4.5–6.2 (F)	5.7–8.5 (M) 3.2–6.0 (F)	5.0–15.0 (M) 3.5–8.0 (F)
MCH (pg)	18.5–21.0	17.5–20.8	18.5–21.0	18.1–20.7	18.0–20.5	18.5–22.0
MCHC (g/dL)	36.6–39.6	35.3–39.2	37.8–40.0	36.9–40.5	37.0–40.6	36.3–40.9
MCV (fl)	48.0–58.0	48.3–56.1	48.0–56.0	47.0–56.0	47.0–56.0	50.0–58.0
Methemoglobin (% Hb)	—	0–3.0	0–4.0	0–2.5	0–2.7	0–2.0
Platelet count ($10^3/\text{mm}^3$)	400–750	350–700	400–870	450–700	450–700	200–450
Reticulocyte count (% RBC)	1.0–2.0	0.7–2.0	0.8–2.0	0.8–2.0	0.3–1.5	0.5–2.5

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; NIEHS, A summary of control values for F344 rats and B6C3F1 mice in 13 week subchronic studies, Program Resources, Research Triangle Park, NC, 1985.

Note: —, data not available.

TABLE 16.16
Mean Control Ranges of Typical Hematology Measurements in B6C3F₁ Mice

Parameters	12–14 Weeks Old	18–20 Weeks Old	32–34 Weeks Old	58–60 Weeks Old	84–86 Weeks Old	110–112 Weeks Old
Erythrocyte count ($10^6/\text{mm}^3$)	9.0–10.2	7.5–10.5	8.0–10.4	8.0–10.0	8.6–10.4	7.7–10.4
Hct (%)	44.1–49.5	36.0–48.6	40.8–46.6	38.5–45.5	40.0–46.9	36.0–43.5
Hb (g/dL)	15.0–17.1	13.1–16.5	15.2–18.2	14.5–17.9	15.0–18.2	13.0–16.8
Leukocyte count, total ($10^3/\text{mm}^3$)	3.0–7.8 (M) 2.5–5.0 (F)	5.5–10.9 (M) 3.2–5.2 (F)	6.1–13.3 (M) 4.2–9.3 (F)	6.1–13.2 (M) 4.6–10.5 (F)	7.0–13.4 (M) 3.9–7.9 (F)	5.0–16.5 (M) 4.2–8.8 (F)
MCH (pg)	16.6–18.8	16.9–20.2	16.4–18.9	15.8–18.0	15.9–18.3	15.7–18.7
MCHC (g/dL)	34.6–38.4	34.6–40.4	37.1–41.2	36.5–39.0	36.2–39.4	35.7–38.8
MCV (fl)	44.0–52.0	45.4–53.6	44.0–48.0	42.0–47.0	42.0–48.0	46.0–50.0
Methemoglobin (% Hb)	—	0–3.0	0–2.5	0–1.5	0–0.9	0–1.0
Platelet count ($10^3/\text{mm}^3$)	700–1100	500–1000	800–1200	700–1200	400–1100	400–800
Reticulocyte count (% RBC)	0.5–2.0	1.0–3.9	0.4–2.8	0.4–1.6	0.2–2.3	0.5–2.5

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; NIEHS, A summary of control values for F344 rats and B6C3F1 mice in 13 week subchronic studies, Program Resources, Research Triangle Park, NC, 1985.

Note: —, data not available.

TABLE 16.17
Mean Control Ranges of Typical Hematology Measurements in CD-1
and BALB/c Mice

Parameters	1–3-Month-Old BALB/c	6–12-Month-Old BALB/c	<1-Year-Old CD-1	>1-Year-Old CD-1
Erythrocyte count ($10^6/\text{mm}^3$)	8.5–10.5	8.8–10.6	8.0–10.0	6.0–9.0
Hct (%)	42.5–47.9	38.3–46.9	36.9–46.9	28.2–41.1
Hb (g/dL)	14.5–16.8	14.2–17.0	13.6–16.8	10.4–14.9
Leukocyte count, total ($10^3/\text{mm}^3$)	2.0–5.7	2.0–5.0	4.0–12.0 (M) 3.5–9.7 (F)	3.4–17.0 (M) 2.4–13.4 (F)
MCH (pg)	15.8–18.4	15.1–17.5	16.1–18.6	15.1–18.4
MCHC (g/dL)	34.2–38.1	35.1–40.6	34.8–38.2	34.6–37.6
MCV (fl)	46.3–50.3	40.9–45.9	44.5–49.7	41.3–51.1
Platelet count ($10^3/\text{mm}^3$)	—	—	700–1400	700–1500
Reticulocyte count (% RBC)	—	—	1.6–3.7	1.7–5.0

Sources: Modified from Frith, C.H. et al., *Lab. Anim. Sci.*, 30, 835, 1980; Wolford, S.T. et al., *Toxicol. Environ. Health*, 18, 161, 1986.

Note: —, data not available.

TABLE 16.18
Mean Control Ranges of Typical Hematology Measurements in Beagle Dogs

Parameters	6–8 Months Old	9–11 Months Old	12–14 Months Old	15–18 Months Old	19–30 Months Old
APTT (s)	9.0–13.0	9.0–13.0	9.0–13.0	9.0–13.0	9.0–13.0
Erythrocyte count ($10^6/\text{mm}^3$)	6.0–7.3	6.2–8.0	6.2–8.2	5.8–7.3	5.8–7.3
Fibrinogen (mg/dL)	150–300	100–200	—	—	—
Hct (%)	41.5–49.0	44.3–54.9	46.0–54.6	42.5–55.0	42.0–52.0
Hb (g/dL)	14.5–17.3	15.8–18.0	16.0–18.8	13.0–19.0	13.0–19.0
Leukocyte count, total ($10^3/\text{mm}^3$)	5.5–14.0	6.8–13.6	5.7–15.5	5.0–15.0	6.0–18.0
MCH (pg)	21.5–25.1	21.6–24.9	22.0–25.2	22.5–26.0	23.0–26.0
MCHC (g/dL)	33.0–37.0	33.0–36.4	34.0–36.0	30.0–34.0	30.0–34.0
MCV (fl)	65.0–71.0	64.0–73.0	64.0–72.0	65.0–78.0	65.0–78.0
Methemoglobin (% Hb)	0–2.0	0–1.5	0–1.5	—	—
Platelet count ($10^3/\text{mm}^3$)	150–400	150–400	150–400	150–400	150–400
PT (s)	6.2–8.4	6.8–8.4	6.2–8.8	6.5–9.0	6.5–9.0
Reticulocyte count (% RBC)	0–0.7	0–0.7	0–0.7	0–0.7	0–0.7

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Bulgin, M.S. et al., *J. Am. Vet. Med. Assoc.*, 157, 1004, 1970; Jordan, J.E., *Am. J. Vet. Res.*, 38, 509, 1977.

Note: —, data not available.

TABLE 16.19**Mean Control Ranges of Typical Hematology Measurements in Nonhuman Primates**

Parameters	3–7-Year-Old Cynomolgus	1–2-Year-Old Rhesus	3–7-Year-Old Rhesus	<1.5-Year-Old Marmoset	>1.5-Year-Old Marmoset	1–5-Year-Old Baboon	6–15-Year-Old Baboon
APTT (s)	15.5–22.7	15.0–22.0	15.0–22.0	—	—	—	30–60
Erythrocyte count (10 ⁶ /mm ³)	4.5–7.2	4.4–5.8	4.2–6.2	4.2–6.2	4.6–6.8	4.2–5.7	4.0–5.3
Hct (%)	31.5–37.9	31.5–39.2	29.3–39.0	30.0–42.1	37.7–47.5	31.0–43.0	34.0–42.0
Hb (g/dL)	10.4–12.4	10.8–13.5	9.8–13.1	12.6–15.0	13.5–16.8	10.8–13.5	10.3–13.1
Leukocyte count, total (10 ³ /mm ³)	5.3–13.4	4.5–13.3	4.3–12.2	5.5–13.0	4.6–11.3	4.9–13.0	4.8–13.9
MCH (pg)	18.9–22.3	19.8–24.8	19.6–23.2	24.0–30.5	23.0–29.0	22.0–27.0	22.0–28.0
MCHC (g/dL)	32.0–35.6	31.3–35.5	31.7–37.5	32.1–42.6	32.2–42.5	28.0–34.0	30.0–35.0
MCV (fl)	57.1–63.9	66.0–74.0	56.0–70.0	66.0–76.0	68.0–77.0	63.0–80.0	75.0–91.0
Platelet count (10 ³ /mm ³)	150–400	200–600	200–500	200–500	200–500	200–500	200–500
PT (s)	11.5–14.0	9.9–12.2	11.2–14.4	—	—	—	9.0–13.0
Reticulocyte count (% RBC)	0–0.5	0–1.4	0–1.5	0–5.0	0–4.7	0–2.3	0–1.9

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Kapeghian, L.C. and Verlangieri, A.J., *J. Med. Primatol.*, 13, 97, 1984; Yarbrough, L.W. et al., *Lab. Anim. Sci.*, 34, 276, 1984; Hack, C.A. and Gleiser, C.A., *Lab. Anim. Sci.*, 32, 502, 1982.

Note: —, data not available.

TABLE 16.20**Mean Control Ranges of Typical Hematology Measurements in New Zealand White Rabbits**

Parameters	15–20 Weeks Old	25–40 Weeks Old	1–2 Years Old
APTT (s)	11.7–14.5	11.3–14.9	10.5–15.8
Erythrocyte count (10 ⁶ /mm ³)	5.5–7.0	4.8–6.7	4.9–7.0
Fibrinogen (mg/dL)	125–300	125–300	125–400
Hct (%)	37.0–44.5	37.0–44.5	37.5–44.7
Hb (g/dL)	12.0–14.7	10.9–14.4	10.5–14.8
Leukocyte count, total (10 ³ /mm ³)	5.4–11.9	3.6–7.9	4.8–13.5
MCH (pg)	20.2–23.0	21.8–24.5	20.4–23.4
MCHC (g/dL)	32.3–34.9	32.2–34.8	30.0–34.1
MCV (fl)	61.4–68.6	64.8–69.5	64.8–72.0
Platelet count (10 ³ /mm ³)	175–500	175–500	200–500
Reticulocyte count (% RBC)	0–2.0	0–2.0	0–3.0
PT (s)	8.2–9.8	8.0–10.0	8.0–10.3

Sources: Modified from Levine, B.S., unpublished data, 1979–1993; Jain, N.C., *Schalm's Veterinary Hematology*, Lea & Febiger, Philadelphia, PA, 1986; Hewett, C.D. et al., *Clin. Chem.*, 35, 1777, 1989.

REFERENCES

1. Altman, P. L. and Dittmer, D. S., *Biology Data Book*, Federation of American Societies for Experimental Biology, Bethesda, MD, 1974.
2. Midgalof, B. H., Methods for obtaining drug time course data from individual small laboratory animals: Serial microblood sampling and assay, *Drug Metab. Rev.*, 5, 295, 1976.
3. Sorg, D. A. and Buckner, B., A simple method of obtaining venous blood from small laboratory animals, *Soc. Exp. Biol. Med. Proc.*, 115, 1131, 1964.
4. Dameron, G. W., Weingand, K. W., Duderstadt, J. M., Odioso, L. W., Dierckman, T. A., Schweske, W., and Baran, K., Effect of bleeding site on clinical laboratory testing of rats: orbital venous plexus versus posterior vena cava, *Lab. Anim. Sci.*, 42, 299, 1992.
5. Meeks, R. G., The rat, in *The Clinical Chemistry of Laboratory Animals*, Loeb, W. F. and Quimby, F. W., Eds., Pergamon Press, New York, 1989, Chapter 2.
6. Riley, J. H. and Cornelius, L. M., Electrolytes, blood gases, and acid base balance, in *The Clinical Chemistry of Laboratory Animals*, Loeb, W. F. and Quimby, F. W., Eds., Pergamon Press, New York, 1989, Chapter 5.
7. Kaneko, J. J., *Clinical Biochemistry of Domestic Animals*, Academic Press, San Diego, CA, 1989.
8. Loeb, W. F. and Quimby, F. W., *The Clinical Chemistry of Laboratory Animals*, Pergamon Press, New York, 1989.
9. Weingand, K., Bloom, J., Carakostas, M., Hall, R., Helfrich, M., Latimer, K., Levine, B. S., Neptun, D., Rebar, A., Stitzel, K., and Troup, K., Clinical pathology testing recommendations for nonclinical toxicity and safety studies, *Toxicol. Pathol.*, 20, 539, 1992.
10. Levine, B. S., unpublished data, 1979–1993.
11. Suber, R. L., Clinical pathology for toxicologists, in *Principles and Methods of Toxicology*, Hayes, A. W., Ed., Raven Press, New York, 1989, Chapter 16.
12. Jain, N. C., *Schalm's Veterinary Hematology*, Lea & Febiger, Philadelphia, PA, 1986.
13. Charles River Laboratories, Serum chemistry parameters for the Crl:CD®BR rat, Wilmington, MA, 1993.
14. NIEHS, A summary of control values for F344 rats and B6C3F1 mice in 13 week subchronic studies, Program Resources, Research Triangle Park, NC, 1985.
15. Burns, K. F., Timmons, E. H., and Poiley, S. M., Serum chemistry and hematological values for axenic (germfree) and environmentally associated inbred rats, *Lab. Anim. Sci.*, 21, 415, 1971.
16. Frith, C. H., Suber, R. L., and Umholtz, R., Hematologic and clinical chemistry findings in control BALB/c and C57BL/6 mice, *Lab. Anim. Sci.*, 30, 835, 1980.
17. Wolford, S. T., Schroer, R. A., Gohs, F. X., Gallo, P. P., Brodeck, M., Falk, H. B., and Ruhren, R. J., Reference range data base for serum chemistry and hematology values in laboratory animals, *Toxicol. Environ. Health*, 18, 161, 1986.
18. Clarke, D., Tupari, G., Walker, R., and Smith, G., Stability of serum biochemical variables from Beagle dogs and Cynomolgus monkeys, *Am. Assoc. Clin. Chem.*, Special Issue, 17, October 1992.
19. Pickrell, J. A., Schluter, S. J., Belasich, J. J., Stewart, E. V., Meyer, J., Hubbs, C. H., and Jones, R. K., Relationship of age of normal dogs to blood serum constituents and reliability of measured single values, *Am. J. Vet. Res.*, 35, 897, 1974.
20. Kaspar, L. V. and Norris, W. P., Serum chemistry values of normal dogs (Beagles): Associations with age, sex, and family line, *Lab. Anim. Sci.*, 27, 980, 1977.
21. Kapeghian, L. C. and Verlangieri, A. J., Effects of primaquine on serum biochemical and hematological parameters in anesthetized *Macaca fascicularis*, *J. Med. Primatol.*, 13, 97, 1984.
22. Davy, C. W., Jackson, M. R., and Walker, S., Reference intervals for some clinical chemical parameters in the marmoset (*Callithrix jacchus*): Effect of age and sex, *Lab. Anim.*, 18, 135, 1984.
23. Yarbrough, L. W., Tollett, J. L., Montrey, R. D., and Beattie, R. J., Serum biochemical, hematological and body measurement data for common marmosets (*Callithrix jacchus*), *Lab. Anim. Sci.*, 34, 276, 1984.
24. Hack, C. A. and Gleiser, C. A., Hematologic and serum chemical reference values for adult and juvenile Baboons (*Papio* sp.), *Lab. Anim. Sci.*, 32, 502, 1982.
25. Hewett, C. D., Innes, D. J., Savory, J., and Wills, M. R., Normal biochemical and hematological values in New Zealand White rabbits, *Clin. Chem.*, 35, 1777, 1989.
26. Yu, L., Pragay, D. A., Chang, D., and Wicher, K., Biochemical parameters of normal rabbit serum, *Clin. Biochem.*, 12, 83, 1979.
27. Charles River Laboratories, Hematology parameters for the Crl:CD®BR rat, Wilmington, MA, 1993.
28. Bulgin, M. S., Munn, S. L., and Gee, S., Hematologic changes to $4\frac{1}{2}$ years of age in clinically normal Beagle dogs, *J. Am. Vet. Med. Assoc.*, 157, 1004, 1970.
29. Jordan, J. E., Normal laboratory values in Beagle dogs at twelve to eighteen months of age, *Am. J. Vet. Res.*, 38, 509, 1977.

APPENDIX: ADDITIONAL RELATED INFORMATION (TABLES 16.A.1 AND 16.A.2)

TABLE 16.A.1
Normal Human Laboratory Values^a

Blood, Plasma, or Serum					
Determination	Reference Value				
	Conventional Units		SI Units		
Ammonia (NH ₃) diffusion	20–120 mcg/dL		12–70 mcmol/L		
Ammonia nitrogen	15–45 µg/dL		11–32 µmol/L		
Amylase	35–118 IU/L		0.58–1.97 mckat/L		
Anion gap (Na ⁺ -[Cl ⁻ + HCO ₃ _ ₋]) (P)	7–16 mEq/L		7–16 mmol/L		
Antinuclear antibodies	Negative at 1:10 dilution of serum		Negative at 1:10 dilution of serum		
Antithrombin III (AT III)	80–120 U/dL		800–1200 U/L		
Bicarbonate					
Arterial	21–28 mEq/L		21–28 mmol/L		
Venous	22–29 mEq/L		22–29 mmol/L		
Bilirubin					
Conjugated (direct)	≤0.2 mg/dL		≤4 mcmol/L		
Total	0.1–1 mg/dL		2–18 mcmol/L		
Calcitonin	<100 pg/mL		<100 ng/L		
Calcium					
Total	8.6–10.3 mg/dL		2.2–2.74 mmol/L		
Ionized	4.4–5.1 mg/dL		1–1.3 mmol/L		
Carbon dioxide content (plasma)	21–32 mmol/L		21–32 mmol/L		
Carcinoembryonic antigen	<3 ng/mL		<3 mcg/L		
Chloride	95–110 mEq/L		95–110 mmol/L		
Coagulation screen					
Bleeding time	3–9.5 min		180–570 s		
Prothrombin time	10–13 s		10–13 s		
Partial thromboplastin time (activated)	22–37 s		22–37 s		
Protein C	0.7–1.4 µ/mL		700–1400 U/mL		
Protein S	0.7–1.4 µ/mL		700–1400 U/mL		
Copper, total	70–160 mcg/dL		11–25 mcmol/L		
Corticotropin (adrenocorticotropic hormone [ACTH])—0800 h	<60 pg/mL		<13.2 pmol/L		
Cortisol					
0800 h	5–30 mcg/dL		138–810 nmol/L		
1800 h	2–15 mcg/dL		50–410 nmol/L		
2000 h	≤50% of 0800 h		≤50% of 0800 h		
Creatine kinase (CK)					
Female	20–170 IU/L		0.33–2.83 mckat/L		
Male	30–220 IU/L		0.5–3.67 mckat/L		
CK isoenzymes, MB fraction	0–12 IU/L		0–0.2 mckat/L		
Creatine	0.5–1.7 mg/dL		44–150 mcmol/L		
Fibrinogen (coagulation factor 1)	150–360 mg/dL		1.5–3.6 g/L		
Follicle-stimulating hormone (FSH)					
Female	2–13 mLU/mL		2–13 IU/L		
Midcycle	5–22 mLU/mL		5–22 IU/L		
Male	1–8 mLU/mL		1–8 IU/L		
Glucose, fasting	65–115 mg/dL		3.6–6.3 mmol/L		
Glucose Tolerance Test (Oral)	mg/dL		mmol/L		
	Normal	Diabetic	Normal	Diabetic	
	Fasting	70–105	>140	3.9–5.8	>7.8
	60 min	120–170	≥200	6.7–9.4	≥11.1
	90 min	100–140	≥200	5.6–7.8	≥11.1
	120 min	70–120	≥140	3.9–6.7	≥7.8

TABLE 16.A.1 (continued)
Normal Human Laboratory Values^a

Blood, Plasma, or Serum		
Determination	Reference Value	
	Conventional Units	SI Units
Gamma glutamyl transferase		
Male	9–50 units/L	9–50 units/L
Female	8–40 units/L	8–40 units/L
Haptoglobin	44–303 mg/dL	0.44–3.03 g/L
Hematologic tests		
Fibrinogen	200–400 mg/dL	2–4 g/L
Hct		
Female	36%–44.6%	0.36–0.446 fraction of 1
Male	40.7%–50.3%	0.4–0.503 fraction of 1
Hemoglobin A _{1c}	5.3%–7.5% of total Hb	0.053–0.075
Hemoglobin		
Female	12.1–15.3 g/dL	121–153 g/L
Male	13.8–17.5 g/dL	138–175 g/L
Leukocyte count (WBC)	3800–9800/mcL	3.8–9.8 × 10 ⁹ /L
Erythrocyte count (RBC)		
Female	3.5–5 × 10 ⁶ /mcL	3.5–5 × 10 ¹² /L
Male	4.3–5.9 × 10 ⁶ /mcL	4.3–5.9 × 10 ¹² /L
MCV	80–97.6 mcm ³	80–97.6 fl
MCH	27–33 pg/cell	1.66–2.09 fmol/cell
MCHC	33–36 g/dL	20.3–22 mmol/L
Erythrocyte sedimentation rate (ESR) (sed rate)	≤30 mm/h	≤30 mm/h
Erythrocyte enzymes	250–5000 units/10 ⁶ cells	250–5000 mcunits/cell
Glucose-6-phosphate dehydrogenase (G-6-PD)		
Ferritin	10–383 ng/mL	23–862 pmol/L
Folic acid: normal	>3.1–12.4 ng/mL	7–28.1 nmol/L
Platelet count	150–450 × 10 ³ /mcL	150–450 × 10 ⁹ /L
Reticulocytes	0.5%–1.5% of erythrocytes	0.005–0.015
Vitamin B ₁₂	223–1132 pg/mL	165–835 pmol/L
Iron		
Female	30–160 mcg/dL	5.4–31.3 mcmol/L
Male	45–160 mcg/dL	8.1–31.3 mcmol/L
Iron-binding capacity	220–420 mcg/dL	39.4–75.2 mcmol/L
Isocitrate dehydrogenase	1.2–7 units/L	1.2–7 units/L
Determination	Reference Value	
	Conventional Units	SI Units
Isoenzymes		
Fraction 1	14%–26% of total	0.14–0.26 fraction of total
Fraction 2	29%–39% of total	0.29–0.39 fraction of total
Fraction 3	20%–26% of total	0.20–0.26 fraction of total
Fraction 4	8%–16% of total	0.08–0.16 fraction of total
Fraction 5	6%–16% of total	0.06–0.16 fraction of total
Lactate dehydrogenase	100–250 IU/L	1.67–4.17 mckat/L
Lactic acid (lactate)	6–19 mg/dL	0.7–2.1 mmol/L
Lead	≤50 mcg/dL	≤2.41 mcmol/L

(continued)

TABLE 16.A.1 (continued)
Normal Human Laboratory Values^a

Determination	Blood, Plasma, or Serum	
	Reference Value	
	Conventional Units	SI Units
Lipase	10–150 units/L	10–150 units/L
Lipids		
Total cholesterol		
Desirable	<200 mg/dL	<5.2 mmol/L
Borderline-high	200–239 mg/dL	<5.2–6.2 mmol/L
High	>239 mg/dL	>6.2 mmol/L
LDL		
Desirable	<130 mg/dL	<3.36 mmol/L
Borderline-high	130–159 mg/dL	3.36–4.11 mmol/L
High	>159 mg/dL	>4.11 mmol/L
HDL (low)	<35 mg/dL	<0.91 mmol/L
Triglycerides		
Desirable	<200 mg/dL	<2.26 mmol/L
Borderline-high	200–400 mg/dL	2.26–4.52 mmol/L
High	400–1000 mg/dL	4.52–11.3 mmol/L
Very high	>1000 mg/dL	>11.3 mmol/L
Magnesium	1.3–2.2 mEq/L	0.65–1.1 mmol/L
Osmolality	280–300 mOsm/kg	280–300 mmol/kg
Oxygen saturation (arterial)	94%–100%	0.94–1 fraction of 1
PCO ₂ , arterial	35–45 mmHg	4.7–6 kPa
PH, arterial	7.35–7.45	7.35–7.45
PO ₂ , arterial: breathing room air ^b	80–105 mmHg	10.6–14 kPa
On 100% O ₂	>500 mmHg	
Phosphatase (acid), total at 37°C	0.13–0.63 IU/L	2.2–10.5 IU/L or 2.2–10.5 mcat/L
Phosphatase alkaline ^c	20–130 IU/L	20–130 IU/L or 0.33–2.17 mcat/L
Phosphorus, inorganic ^d (phosphate)	2.5–5 mg/dL	0.8–1.6 mmol/L
Potassium	3.5–5 mEq/L	3.5–5 mmol/L
Progesterone		
Female	0.1–1.5 ng/mL	0.32–4.8 nmol/L
Follicular phase	0.1–1.5 ng/mL	0.32–4.8 nmol/L
Luteal phase	2.5–28 ng/mL	8–89 nmol/L
Male	<0.5 ng/mL	<1.6 nmol/L
Prolactin	1.4–24.2 ng/mL	1.4–24.2 mcg/L
Prostate specific antigen	0–4 ng/mL	0–4 ng/mL
Protein		
Total	6–8 g/dL	60–80 g/L
Albumin	3.6–5 g/dL	36–50 g/L
Globulin	2.3–3.5 g/dL	23–35 g/L
Rheumatoid factor	<60 IU/mL	<60 kIU/L
Sodium	135–147 mEq/L	135–147 mmol/L
Testosterone		
Female	6–86 ng/dL	0.21–3 nmol/L
Male	270–1070 ng/dL	9.3–37 nmol/L
Thyroid hormone function tests		
Thyroid-stimulating hormone (TSH)	0.35–6.2 mcU/mL	0.35–6.2 mU/L
Thyroxine-binding globulin capacity	10–26 mcg/dL	100–260 mcg/L

TABLE 16.A.1 (continued)
Normal Human Laboratory Values^a

Blood, Plasma, or Serum		
Determination	Reference Value	
	Conventional Units	SI Units
Total triiodothyronine (T ₃)	75–220 ng/dL	1.2–3.4 nmol/L
Total thyroxine by RIA (T ₄)	4–11 mcg/dL	51–142 nmol/L
T ₃ resin uptake	25%–38%	0.25–0.38 fraction of 1
Aspartate aminotransferase (AST)	11–47 IU/L	0.18–0.78 mckat/L
Alanine aminotransferase (ALT)	7–53 IU/L	0.12–0.88 mckat/L
Transferrin	220–400 mg/dL	2.20–4.00 g/L
Urea nitrogen (BUN)	8–25 mg/dL	2.9–8.9 mmol/L
Uric acid	3–8 mg/dL	179–476 mcmol/L
Vitamin A (retinol)	15–60 mcg/dL	0.52–2.09 mcmol/L
Zinc	50–150 mcg/dL	7.7–23 mcmol/L
Urine		
Determination	Reference Value	
	Conventional Units	SI Units
Calcium ^c	50–250 mcg/day	1.25–6.25 mmol/day
Catecholamines		
Epinephrine	<20 mcg/day	<109 nmol/day
Norepinephrine	<100 mcg/day	<590 nmol/day
Catecholamines, 24 h	<110 µg	<650 nmol
Copper ^c	15–60 mcg/day	0.24–0.95 mcmol/day
Creatinine		
Child	8–22 mg/kg	71–195 µmol/kg
Adolescent	8–30 mg/kg	71–265 µmol/kg
Female	0.6–1.5 g/day	5.3–13.3 mmol/day
Male	0.8–1.8 g/day	7.1–15.9 mmol/day
pH	4.5–8	4.5–8
Phosphate ^c	0.9–1.3 g/day	29–42 mmol/day
Potassium ^c	25–100 mEq/day	25–100 mmol/day
Protein		
Total	1–14 mg/dL	10–140 mg/L
At rest	50–80 mg/day	50–80 mg/day
Protein, quantitative	<150 mg/day	<0.15 g/day
Sodium ^c	100–250 mEq/day	100–250 mmol/day
Specific gravity, random	1.002–1.030	1.002–1.030
Uric acid, 24 h	250–750 mg	1.48–4.43 mmol

Source: Reprinted from *Drug Facts and Comparisons. 2000 Loose Leaf Edition*, Facts and Comparisons, St Louis, MO, 2000. Copyright 2000. With permission.

^a In this table, normal reference values for commonly requested laboratory tests are listed in traditional units and in SI units. The table is a guideline only. Values are method dependent and “normal values” may vary between laboratories.

^b Age dependent.

^c Infants and adolescents up to 104 IU/L.

^d Infants in the 1st year up to 6 mg/dL.

^e Diet dependent.

TABLE 16.A.2
Erythrocyte Life Span in Various Animals^a

Species	Mean Life Span ^b (Days)
Man	117–127 (120) ^c
Dog	90–135
Cat	66–79
Pig	62–86
Rabbit	50–80
Guinea pig	70–90
Hamster	60–70
Rat	50–68 (60) ^c
Mouse	41–55

^a Determined by use of isotopes.

^b Range of means from various studies.

^c Most often cited.

17 Metabolism and Toxicokinetics of Xenobiotics

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INTRODUCTION

Xenobiotics entering biological systems undergo the following processes: I, absorption: oral, dermal, inhalation; II, tissue distribution (disposition); III, protein and tissue binding; IV, enzymatic and nonenzymatic chemical transformation; and V, excretion.

Pharmacokinetics is defined as the rates of all metabolic processes related to the expression of pharmacology.

Toxicokinetics is defined as the rates of all metabolic processes related to the expression of toxicological end points.

The purpose of pharmacokinetics or toxicokinetics: (1) to allow predictions of body burdens, both time to maximum and amount; (2) to allow prediction of duration in body after exposure terminated; (3) to determine percent absorbed; and (4) to determine schedule of drug dosage.

ABSORPTION, DISTRIBUTION AND ELIMINATION

DRUG ADMINISTRATION

A drug is administered to reach a therapeutic level in plasma and subsequent absorption in tissues.

1. Enteral routes

a. Sublingual (buccal)

The drug is placed beneath the tongue. Absorbed drug in blood vessels in the mouth bypasses the liver, does not undergo first-pass liver metabolism, and reaches directly the systemic circulation. This route is mostly used for nitrates and certain hormones.

b. Oral

This is the most common route of drug administration (Abou-Donia, 1976; 1980). Orally administered drugs undergo liver and intestinal first-pass metabolism. Bioavailability of orally administered drugs follows this order: solution > suspension > capsule > tablet > coated tablet.

c. Rectal

Absorption of drugs is slow because the rectum is devoid of villi. This route is mostly used in small children.

2. Parenteral routes

a. Intravenous (IV) injection

This route is used when rapid clinical action is desired. Injection should be over 1–2 min or at slow IV infusion or drip.

b. Intra-arterial (IA) injection

This route is used to deliver a high concentration of drug to a particular tissue. The injected artery leads a drug, example, anticancer, directly to target organ.

c. Intrathecal (IT) injection

Because blood–brain barrier (BBB) prevents many drugs into cerebrospinal fluid, in certain situations, a drug is administered via lumbar puncture and injection into subarachnoid space.

d. Intramuscular (IM) injection

Because of differences in vascularity, the rates of absorption follow this order: arm > thigh > buttocks. Drug absorption may be slow and erratic and is affected by osmolality of the solution, lipid solubility, and degree of ionization.

e. Subcutaneous (SC) injection

Because of poor vascularity, SC is slower than IM.

f. Inhalation

Absorption of volatile chemicals via inhalation is rapid because of the large alveolar surface area and blood supply. Drug administered do not undergo first-pass liver metabolism.

g. Topical application

i. Eye

ii. Intravaginal: for contraceptives

iii. Intranasal

iv. Skin (Maibach et al., 1971)

v. Drug patches

Drug enters systemic circulation by zero-order kinetics (a constant amount of drug enters the circulation per unit time).

ABSORPTION

In order for a xenobiotic to reach its site of action, it must pass across various body membranes, that is, cells of skin, cells of lung, gastrointestinal tract, erythrocyte membrane, etc.

1. Membrane structure

a. Phospholipid bilayer

b. Embedded with proteins

c. Contains various size pores

2. Mechanisms by which xenobiotics pass body membranes

a. Passive transport

i. Simple diffusion

(1) This is the mechanism by which most chemicals pass membranes. It occurs through channels only for molecules of mass <150–200 kDa. An exception is the endothelial capillary lining with large pores that allow molecules of 20,000–30,000 kDa to pass. In contrast, capillaries of most of the brain lack these large pores. The brain is protected by the BBB that is formed by brain capillary walls endothelial cells that are enforced (see later.)

(2) Nonsaturable process.

(3) Both lipid-soluble substances and lipid-insoluble molecules of small size (i.e., urea) may cross body membranes by simple diffusion.

(4) Many xenobiotics (weak organic acids and bases) exist in solution in both the

ionized and unionized forms. The ionized form is often unable to penetrate the cell membrane because of its low lipid solubility, while in contrast, the unionized form may be lipid-soluble enough to diffuse across cell membranes.

- (5) The amount of weak organic acid or base in the unionized form is dependent on its association constant (pK_a) and the pH of the internal environment (Tables 17.1 through 17.3). This relationship is described by the Henderson-Hasselbalch equation:

$$\text{For acids: } pK_a - \text{pH} = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$$

$$\text{For bases: } pK_a - \text{pH} = \log \frac{[\text{ionized}]}{[\text{nonionized}]}$$

For Weak Acids:	For Weak Bases:
$pK_a - \text{pH} = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$	$pK_a - \text{pH} = \log \frac{[\text{ionized}]}{[\text{nonionized}]}$
Benzoic acid $pK_a \approx 4$	Aniline $pK_a \approx 5$
Stomach pH ≈ 2	Stomach pH ≈ 2
$4 - 2 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$	$5 - 2 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$
$2 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$	$3 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$
$100 = \frac{[\text{nonionized}]}{[\text{ionized}]}$	$1000 = \frac{[\text{nonionized}]}{[\text{ionized}]}$
Ratio favors absorption	
Intestine pH ≈ 6	Intestine pH ≈ 6
$4 - 6 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$	$5 - 6 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$
$-2 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$	$-1 = \log \frac{[\text{nonionized}]}{[\text{ionized}]}$
$\frac{1}{100} = \frac{[\text{nonionized}]}{[\text{ionized}]}$	$\frac{1}{10} = \frac{[\text{nonionized}]}{[\text{ionized}]}$
	Ratio favors absorption

ii. Filtration

When water flows in bulk across a porous membrane, any solute that is small enough to pass through the pores flows with it.

Pores in most cells are 4 Å, endothelial cells of capillaries are 40 Å, and endothelial cells of glomerular membrane of the kidney are 100 Å.

b. Special transport:

There are three types of special transport:

- i. *Active transport*: It occurs when substances are moving against a concentration or electrochemical gradient. It has the following characteristics:

- (1) Compounds move against concentration or electrical gradient
- (2) Can be saturated
- (3) Selectivity for compounds of the same size
- (4) Competitive inhibition among substances handled by the same mechanism
- (5) Requires energy (metabolic inhibitors block transport process)

- ii. *Facilitated transport*: Similar to active transport, however, in this type, chemicals do not move against a concentration gradient and does not require energy

- iii. *Pinocytosis*: Refers to the ability of cells to engulf small droplets

3. Absorption of xenobiotic in the body

a. Gastrointestinal tract

- i. Lipid-soluble compounds (nonionized) are more readily absorbed than water-soluble compounds (water-soluble, ionized)

- (1) Weak organic bases are in the nonionized lipid-soluble form in intestine and tend to be absorbed there.
- (2) Weak organic acids are in the nonionized lipid-soluble form in the stomach and are absorbed there, but intestine is more important because of time and area of exposure.

- ii. In general, the absorption of chemicals from the gastrointestinal tract is by simple diffusion.

- iii. The following chemicals are absorbed through specialized transport systems: sugars, amino acids, pyrimidines, calcium, and sodium ions.

- iv. Almost everything is absorbed at least to a small extent.

- v. Blood flow rate and surface area are more important than pH for the absorption of weak acids (Table 17.4).

- vi. Effect of digestive fluids on chemicals:

- (1) Nitrites plus secondary amines from nitrosamines (carcinogens)
- (2) Intestinal flora degrade DDT to DDE

- vii. Dilution—increases toxicity because of more rapid absorption from intestine.

- viii. Age—newborn has poor intestinal barrier

- ix. "First pass"—chemical can be extracted and/or biotransformed by intestine or liver before it reaches systemic circulation. This may be a protective mechanism in toxicology but not desirable in pharmacology.
- b. Lungs
 - i. Aerosol deposition
 - (1) Nasopharyngeal—5 μm or larger
 - (2) Tracheobronchiolar—1 to 5 μm
 - (3) Alveolar—1 μm
 - ii. Mucociliary transport
 - iii. Anatomically good for absorption
 - (1) Large surface area (50–100 m^2)
 - (2) Blood flow is high
 - (3) Close to blood (10 μm)
 - iv. Absorption of gases is dependent on solubility of gas in blood
 - (1) Chloroform—high solubility in blood—all absorbed—respiration limited
 - (2) Ethylene—low solubility—small percentage is inhaled
- c. Skin
 - i. Skin is a relatively good barrier (Feldman and Maibach, 1970).
 - ii. Absorption through follicles is rapid.
 - iii. Absorption transdermally is quantitatively important.
 - iv. With most compounds, the surface loss rate must exceed the absorption rate, for the latter is generally below 50%.
 - v. Absorption by passive diffusion, example, high lipid solubility of a chemical should favor its absorption.
 - vi. Diffusion barrier is stratum barrier.
 - vii. Abrasion increased absorption.
- d. Relative rates of absorption: IV injection > inhalation > intraperitoneal injection > oral > dermal

TABLE 17.1
Calculation of the Extent of Ionization,
given pK_a and pH

pK_a -pH	Percent Ionized (if Anion)	Percent Ionized (if Cation)
-4	99.99	0.01
-3	99.94	0.10
-2	99.01	0.99
-1	90.91	9.09
0	50.00	50.00
1	9.09	90.91
2	0.99	99.01
3	0.10	99.94
4	0.01	99.99

TABLE 17.2
 pK_a Values for Some Weak Acids and Bases
(at 25°C)

Weak Acids	pK_a	Weak Bases	pK_a
Salicylic acid	3.00	Reserpine	6.6
Acetylsalicylic acid	3.49	Codeine	7.9
Sulfadiazine	6.48	Quinine	8.4
Barbital	7.91	Procaine	8.8
Boric acid	9.24	Ephedrine	9.36
		Atropine	9.65

TABLE 17.3
Effect of pH on the Ionization of Salicylic Acid
($pK_a = 3$)

pH	Nonionized
1	99.0
2	90.9
3	50.0
4	9.09
5	0.99
6	0.10

TABLE 17.4
Blood Flow Rate and Surface Area

Tissue	pH	Blood Flow Rate (L/min)	Surface Area (m^2)
Stomach	1–3	0.15	1
Intestine	5–8	1.0	200

DISTRIBUTION OF TOXICANTS

1. Factors affecting distribution of chemicals
 - a. Blood flow through the organ
 - b. Passage across cell membranes
 - c. Affinity of various tissues for the toxicant
2. Site of concentration in body is not necessarily the target organ of toxicity
3. Plasma proteins as a storage depot for toxicants
 - a. Toxicants become biologically inactive when they bind to plasma proteins.
 - b. Bound toxicants cannot be filtered at the kidney.
 - c. A bound chemical can displace another.
 - d. The binding capacity of a protein is not unlimited. Once it becomes saturated, a sudden increase in toxicity occurs with further absorption of toxicants.
 - e. The protein responsible for binding is usually albumin, although globulins may be very important in binding of some hormonal agents and pesticides.
4. Fat as a storage depot
 Nonionized lipid-soluble xenobiotics, that is, DDT tend to concentrate in adipose tissue.
5. Bone as a storage depot
 For lead, strontium, and fluoride

6. Cellular binding

This usually results from the affinity of a substance to some cellular component. An example is the high concentrations of alkaloids in the liver and muscle which is attributed to the affinity of these naturally occurring amines to nucleoproteins.

7. Blood–brain barriers

The brain is protected by the BBB, which can only be penetrated by lipid-soluble substances; some molecules can cross BBB via specialized carriers that are energy-dependent. After passing this barrier, a xenobiotic still has to penetrate the membranes of the brain. BBB is formed by brain capillary walls' endothelial cells characterized by the presence of

- (1) Tight junctions
- (2) No fenestras
- (3) Few pinocytotic vesicles in the cytoplasm (BChE)
- (4) Increased mitochondria (active transport)
- (5) Basement membrane (AChE)
- (6) Astrocytic feet ensheath 95% of endothelial cells
- (7) Pericytes with smooth muscle-like properties
- (8) P-glycoprotein (P-gp) to remove undesired substances

8. Placenta barrier

- a. Placenta is a poor barrier—virus (Rubella), cellular pathogens (syphilis), antibody, globulins, and erythrocytes are transported.
- b. Transport is by diffusion.

BINDING

Binding of xenobiotics to proteins and other micromolecules in the body significantly affects their distribution, metabolism, and elimination (Abou-Donia et al., 1990). Albumin is the major protein that xenobiotics bind to followed by globulins. Both anions and cations bind to plasma albumins. Bound chemicals may displace each other. Binding to plasma proteins renders them biologically inactive and less available for filtering by kidney. When plasma proteins become saturated further, absorption of toxicants may result in toxicity. Drug binding to plasma proteins is generally weak and rapidly reversible; thus, protein-bound drug can be considered to be a temporary storage compartment.

Some xenobiotics have greater affinity for body tissues than plasma proteins (Brodie and Axelrod, 1948). Alkaloids, the naturally occurring amines, have high affinity for nucleoproteins resulting in their high concentrations in the liver. Furthermore, some metals and organic amines are transferred to liver after their initial binding to plasma. Bones and teeth act as depots for some inorganic ions such as fluoride, lead, and strontium as well as drugs such as tetracycline. On the other hand, lipid-soluble xenobiotic such as dichlorodiphenyltrichloroethane (DDT) and leptophos tend to concentrate in adipose tissue.

Lipid-soluble xenobiotics cross the placenta and are deposited in the fetus. This process takes place via passive diffusion. Organophosphorus insecticides, example, methamidophos, acephate, and chlorpyrifos cross the placenta in

pregnant rats (Bakry et al., 1990). Virus, example, rubella, bacteria, example, syphilis, antibodies, globulins, and erythrocytes can also cross the placenta.

EXCRETION OF TOXICANTS

Routes and Mechanisms of Excretion

The kidney is the most important organ by which xenobiotics and/or their metabolites are excreted (Abu-Qare and Abou-Donia, 2000a,b). Other routes of excretions are bile, sweat, exhaled air, and milk.

1. Kidney

Two major mechanisms are involved in the renal handling of xenobiotics: (1) glomerular filtration with variable tubular reabsorption and (2) tubular secretion (an active transport mechanism). Usually, the xenobiotic is filtered from the blood through the highly porous glomeruli and is partially reabsorbed by the tubules, depending on its lipid solubility property. Lipid-soluble compounds are reabsorbed from the tubules by nonionic diffusion. The tubular epithelium of the distal convoluted tubule is selectively permeable or more permeable to the unionized lipid-soluble molecule than the less lipid-soluble corresponding anion or cation. Generally xenobiotics that are bases are excreted to a greater extent if the urine is acid (as salts), whereas acid compounds are excreted more favorably if the urine is alkaline (as salts) (Milne et al., 1958).

a. Excretion into renal tubule

i. Glomerular filtration

- (1) All water-soluble toxicants with MW <60,000 that are not protein-bound are filtered from the blood through the highly porous glomeruli.

ii. Passive tubular diffusion

- (1) If lipid-insoluble
- (2) If weak acid or weak base:
 - (a) Weak acidic chemicals excreted better in alkalized urine.
 - (b) Weak basic chemicals excreted better in acidic urine.

iii. Active secretion

- (1) It is carrier-mediated, with two separate carriers:
 - (a) Organic acids, example, *p*-aminohippurate
 - (b) Organic bases, example, *N*-methylnicotinamide
- (2) Characteristic of carriers
 - (a) Rate of excretion is not affected by binding to plasma proteins
 - (b) Competition for excretion

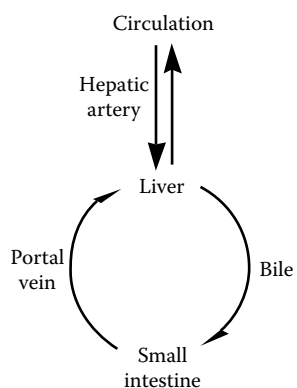
b. Passive tubular reabsorption influenced by

- i. Lipid solubility
- ii. pK_a
- iii. Tubular fluid pH
- iv. Tubular fluid volume

2. Bile

A xenobiotic in circulation enters the liver via the hepatic artery or through the lymph. The compound emerges from the liver (as such or in a degraded state) with the bile, which passes down the bile duct into the gall bladder, which exits for bile storage (Baker and Bradley, 1966). At intervals, bile leaves the gall bladder by the bile duct, which discharges into the duodenum. Some xenobiotics are recycled by reabsorption from the small intestine by the portal vein and then into the liver, which secretes again into the small intestine. Both circuits involve a gradual decrement via the feces.

- a. Classification of toxicants excreted into the bile
 - i. Class A: Bile/plasma concentration ratio = 1
Examples: sodium, potassium, mercury, etc.
 - ii. Class B: Bile/plasma concentration ratio >1 (10–100)
Examples: bile salt, lead, manganese, weak acids, etc.
 - iii. Class C: Bile/plasma concentration ratio <1
Examples: inulin, albumin, iron, etc.
- b. Mechanisms of excretion into the bile
 - i. Diffusion
 - ii. Carrier-mediated transport (Class B)
 - (1) Organic acid—Bromosulfthalein (BSP)
 - (2) Organic base—Procainamide ethyl bromide (PAEB)
 - (3) Organic neutral—Ouabain
- c. Factors favoring biliary excretion of weak acids
 - i. Molecular weight >325
 - ii. Two or more aromatic rings
 - iii. The presence of polar groups
 - iv. Highly protein-bound substances in plasma are readily transferrable directly to the secreting cells
 - v. Microsomal induces enhance biliary secretion
- d. Enterohepatic circulation
Cholestyramine enhances biliary excretion of ketone (Williams, 1965)



- e. The following species do not have gall bladder: rat, horse, and deer

3. Lung

- a. Important for
 - i. Substances that exist in gas phase at body temperature
 - ii. Liquids
- b. Mechanisms of elimination—diffusion
- c. Rate of excretion
 - i. Low blood/gas solubility—rapid example, ethylene
 - ii. High blood/gas solubility—slow example, chloroform
4. Gastrointestinal tract
Sources of toxicants in feces
 - a. Not completely absorbed following ingestion
 - b. Excreted into bile
 - c. From respiratory tract is swallowed
 - d. Excreted in saliva, pancreatic, gastric, or intestinal secretions
5. Cerebrospinal fluid
 - a. With bulk flow of cerebrospinal fluid
 - b. Diffusion from brain to blood
 - c. Active transport at choroid plexus
6. Milk
 - a. Lipid-soluble, nonionized nonprotein-bound chemicals pass into milk by diffusion.
 - b. Toxic material may be passed from mother to nursing child.
 - c. Compounds may be passed from cows to humans.
 - d. Toxicants that have been detected in human and cows' milk: DDT, polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB).
7. Sweat and saliva
8. Exhaled air
9. Placental transfer (Abu-Qare and Abou-Donia, 2000a,b)

METABOLIC BIOTRANSFORMATION OF XENOBIOTICS

Metabolic transformations of xenobiotics are the changes of these chemicals produced by biological environments. The alterations convert these chemicals into more polar and water-soluble derivatives that may be readily excreted.

ORGAN SITES OF DRUG METABOLISM

Xenobiotic-metabolizing enzymes occur in all tissues with the liver having the largest amount (Carrington and Abou-Donia, 1988). The gastrointestinal tract is the most important extrahepatic site. Drugs that are conjugated extensively in the intestine suffer decrease in their bioavailability. The lung, kidney, intestine, skin, and placenta can also metabolize drugs (Chabra et al., 1974). The relative amounts of these enzymes in tissues are listed in Table 17.5.

TABLE 17.5

Tissue Localization of Xenobiotic-Metabolizing Enzymes

Relative Amount	Tissue
High	Liver
Medium	Lung, kidney, intestine
Low	Skin, testes, placenta, adrenals
Very low	Nervous system tissues

CELLULAR SITES OF DRUG METABOLISM

The lipophilic membranes of the smooth endoplasmic reticulum (SER) contain most drug-metabolizing enzymes. SER is isolated from tissues via differential centrifugation in the cellular fraction known as “microsomal fraction.”

BIOCHEMICAL SYSTEMS OF DRUG METABOLISM

Metabolic biotransformation reactions of xenobiotics in biological systems (Abou-Donia and Nomeir, 1986) are divided into two phases:

Phase I: Phase I reactions modify xenobiotics to undergo Phase II conjugation reactions. Phase I reactions are listed in Table 17.6.

Phase I reactions introduce polar groups into the chemical that undergoes these reactions including carboxyl, epoxide, hydroxyl, sulfhydryl, amine, hydroxylamine, and imine (Fouts and Brodie, 1957). Some of the metabolites produced by reactions in this phase are more reactive than their parent compounds. This may result in covalent binding with critical targets leading to toxic reactions (Mason, 1957). On the other hand, most of these polar metabolites undergo conjugation through Phase II reactions listed in Table 17.6. These conjugated products are more polar and readily excreted via biliary or urinary routes.

Phase II: Reactions in this phase result in the introduction of polar, acidic endogenous functional groups that usually render Phase II reaction products more polar, less lipid-soluble, more strongly acidic, and therefore more easily excreted. Metabolic pathways are affected by general factors such as the intrinsic properties of the chemical, dose, animal species, strain, sex, and age, as well as other environmental considerations.

TABLE 17.6

Metabolic Phase I and Phase II Reactions

Phase I	Phase II
Oxidation	Glucuronidation
Reduction	Glucosidation
Hydrolysis	Ethereal sulfation
Isomerization	Methylation
Others	Acetylation
	Amino acid conjugation
	Glutathione conjugation
	Fatty acid conjugation
	Condensation

XENOBIOTIC-METABOLIZING REACTIONS: PHASE I**MICROSOMAL MIXED-FUNCTION OXIDASE (MFO, CYTOCHROME P450)**

The mixed-function oxidase enzyme system is an iron-containing porphyrin protein (Conner, 1967). The term “cytochrome P450” is used because the reduced hemoprotein, with the iron moiety in its ferrous (Fe^{2+}) oxidation state, combines with carbon monoxide to form a ligand with a maximal absorbance at 450 nm (Dutton, 1971). This property takes place only when enzyme is intact and catalytically functional. When denatured, cytochrome P450 loses its peak at 450 nm and produces only a 420 nm absorbance maximum (Omura and Sato, 1964). Notably, the nomenclature committee of the International Union of Biochemistry proposed the term “heme–thiolate protein” instead of “cytochrome” for P450 (Guengerich, 1992).

1. Isolation of MFO system

These enzymes are localized in the SER of cells of most mammalian tissues (Waxman et al., 1983). Homogenization of liver tissue followed by differential centrifugation separates the endoplasmic reticulum in the microsomal fraction (Kaminsky et al., 1981). A 10% homogenate of liver in 0.25 M sucrose– 10^{-3} M EDTA is prepared in a Waring blender at a full speed for 30 s. The homogenate is subjected to centrifugation at 600 g for 15 min to sediment nuclei, red cells, and cell debris. The supernatant is then centrifuged at 15,000 g for 30 min followed by centrifugation at 105,000 g for 60 min to sediment the microsomal fraction from the supernatant or soluble fraction. The microsomal fraction is heterogeneous in the following aspects: (a) morphologically, because it consists of different organelles: SER, rough endoplasmic reticulum, ribosomes, and Golgi apparatus, (b) physiologically, because it performs various physiological functions, and (c) biochemically, because it contains many enzymes with diverse catalytic activities (La Du et al., 1972).

2. Classification of cytochrome P450

Early studies have demonstrated that cytochrome P450 exists in more than one form. Thus, animal treatment with certain chemicals, example, 3-methyl cholanthrene and β -naphthoflavone, caused a shift in the spectral maximum of cytochrome P450–P448. Recent studies isolated and characterized cytochrome P450 isozymes. These isozymes differ in both (a) the structure of the polypeptide chain and (b) the specificity of the reaction they catalyze. The types and amounts of cytochrome P450 vary with species, organ, age, health, sex, strain, and chemical exposure.

Cytochrome P450 isozymes are grouped into families based on ~40% homology of amino acid sequence in any gene family (Nebert and Gonzalez, 1987). Gene families are further divided into subfamilies which are >59% identical. Gene families 1, 2, 3, and 4 consist of hepatic and extrahepatic cytochrome P450 isozymes involved in Phase I biotransformation reactions. Gene families 9, 17, 19, and 21 consist of extrahepatic cytochrome P450s involved in steroid hormone biosynthesis.

Recently, a total of 221 cytochrome P450 and 12 putative pseudogenes have been characterized (Nelson et al., 1993). These genes belong to 31 eukaryotes (including 11 mammalian and 3 plant species) and 11 prokaryotes. Only 12 families exist in all mammals examined among 36 gene families described so far. The 12 mammalian families comprise 22 mammalian subfamilies. The recommended nomenclature for the gene and cDNA is as follows: the italicized root symbol *CYP* for human (*Cyp* for mouse), representing cytochrome P450, is followed by an Arabic numeral denoting the family, a letter designating the subfamily, and an Arabic numeral representing the individual gene within the subfamily. A hyphen should precede the final number in mouse genes. If a gene is the only member of a family, the subfamily letter and gene number are not included. Tables 17.7 and 17.8 summarize the classification of cytochrome P450 gene families in man and some animal species that are briefly described as follows:

Cytochrome P450 subfamilies

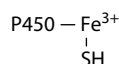
- 1) The CYP1A subfamily is highly observed among mammalian species. CYP1A1 retain activity toward polycyclic aromatic hydrocarbons and CYP1A2 retain activity toward aromatic and heterocyclic amines. In human, CYP1A1 is expressed only after exposure to inducers. Cigarette smoking has been shown to induce the CYP1A1 protein in placenta and in lung tissues. CYP1A2 is present in most human livers.
- 2) The CYP1B subfamily was recognized as being TCDD-inducible in human keratinocytes, but they may be present in other tissues after exposure to inducers.
- 3) The CYP2A subfamily: The CYP2A1 is expressed in the liver. Its role in the xenobiotic metabolism has not been extensively investigated. CYP2A6 is expressed in the liver that its hepatic level varies from very high to not detectable.
- 4) The CYP2B subfamily: CYP2B1 is expressed in liver and extrahepatic tissues. CYP2B6 is

variably expressed in human liver specimen. It activates aflatoxin B1.

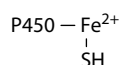
- 5) The CYP2C subfamily enzymes are expressed in human liver and are present at lower levels in the small intestine. They are comprised of human CYP2C9, human CYP2C18, human CYP2C19, and rat CYP2C.
 - 6) The CYP2D subfamily: Among them is CYP2D1 that is capable of metabolizing drugs such as bufuralol and debrisoquine in rat and human (Teh and Bertilsson, 2012). CYP2D6 is responsible for common genetic defect in the oxidation of many drugs. Quinidine is a potent inhibitor of this enzyme.
 - 7) The CYP2E subfamily: CYP2E1 is constitutively expressed in human liver and subject to induction by a variety of mechanisms. Is capable of activating the procarcinogens N-nitrosodimethylamine and N-nitrosodiethylamine.
 - 8) The CYP3A subfamily: CYP3A4 is the most abundant cytochrome P450 in human liver. It plays an important role in the metabolism of drugs. CYP3A3 and CYP3A4 are similar in sequence and expressed in livers of most adults. CYP3A5 is expressed in about 20% of human adult liver samples. CYP3A7 is expressed in human fetal liver and kidney samples.
 - 9) The CYP4A subfamily: This subfamily has not been extensively studied in humans. They are catalyzed the ω -hydroxylation of lauric acid.
3. Mechanisms of cytochrome P450-mediated reactions
- Cytochrome P450 consists of (Figure 17.1):
- a. Two flavoproteins (dehydrogenases), that is, NADPH cytochrome P450 reductase and NADH cytochrome b5 reductase
 - b. Two hemoproteins, that is, cytochrome P450 and cytochrome b5
 - c. Two pyridine nucleotides, that is, NADH and NADPH
- These enzymes are embedded in the phospholipid matrix of the endoplasmic reticulum. The phospholipids facilitate the interactions between the two enzymes. These components function as follows:
1. The flavoproteins functions as dehydrogenases by transferring an electron from NADPH to cytochrome P450 and from NADH to cytochrome b₅.
 2. The NADPH cytochrome P450 reductase donates the first of two electrons to cytochrome P450 and cytochrome b₅ transfers the second electron to cytochrome P450.
4. Mechanism of oxidation reactions
- Cytochrome P450-mediated reactions require both NADPH and oxygen to oxidize a substrate

(SH) (Figure 17.2). This reaction follows the following steps:

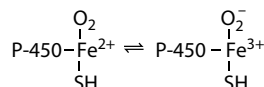
Step 1: The substrate (SH) binds to the iron atom in the oxidized state (Fe^{3+}) at the active site of cytochrome P450:



Step 2: Cytochrome P450–substrate complex is reduced with an electron transferred from NADPH cytochrome reductase:

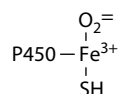


Step 3: The reduced cytochrome P450–substrate complex then binds to a molecular oxygen



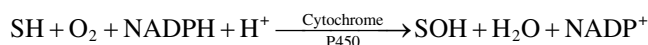
Step 4: The cytochrome P450–substrate–oxygen complex is then further reduced by a second electron, possibly donated by

- Cytochrome b_5 which receives it from NADH by cytochrome b_5 reductase
- NADH cytochrome b_5 reductase



Step 5: The cytochrome P450–substrate–oxygen complex splits into

- Water
 - Oxidized substrate (SOH)
 - Oxidized form of cytochrome P450 ($\text{P450}-\text{Fe}^{3+}$)
- The overall reaction is therefore presented by the following equation:



5. Cytochrome P450–mediated reactions

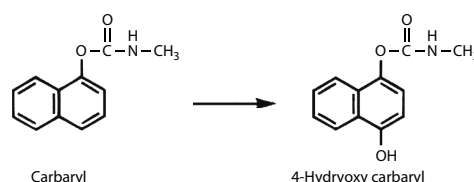
Cytochrome P450 enzymes mediate numerous reactions including the oxidative, peroxidative, reductive, and hydrolytic metabolism of endogenous substrates, example, steroids, bile acids, fatty acids,

prostaglandins, leukotrienes, and biogenic amines. Also, the metabolism of many xenobiotics including drugs, industrial chemicals, pollutants, and natural products are metabolized with these enzymes (Othman and Abou-Donia, 1988).

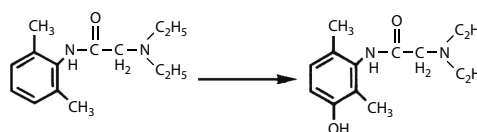
a. Oxidation reactions

i. Aromatic hydroxylation

This reaction is common for aromatic ring-containing xenobiotics. For example, the carbamate insecticide, carbaryl, is oxidized to produce 4-hydroxy carbaryl.



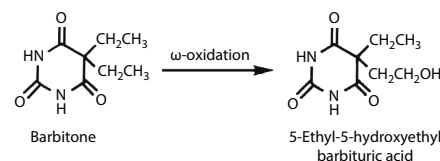
Another example is the oxidation of the local anesthetic drug, lignocaine, to the 3-hydroxy derivative.



ii. Aliphatic hydroxylation

Alkyl chains may be oxidized to form primary, secondary, or tertiary alcohols depending upon the location of the oxidized carbon atom. Oxidation may occur at the ω , $\omega-1$, or tertiary carbon atom.

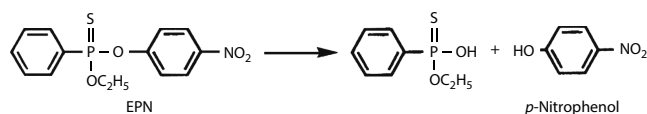
(1) ω -Oxidation. ω -Oxidation is a common oxidation reaction of aliphatic side chains such as in barbitone.



Another example is the hydroxylation of a methyl group attached to aromatic or heterocyclic ring systems:

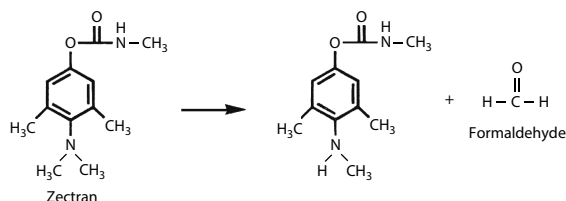


EPN, a phenylphosphonothioate insecticide, is dearylated to yield *p*-nitrophenol.

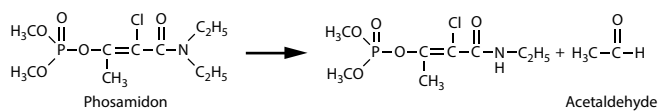


(2) N-Dealkylation

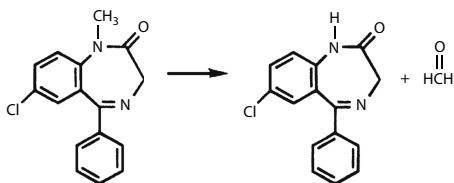
This reaction proceeds in two steps similar to O-demethylation. An example is the N-demethylation of the carbamate insecticide Zectran.



Similarly, N-deethylation occurs with the insecticide phosamidon.

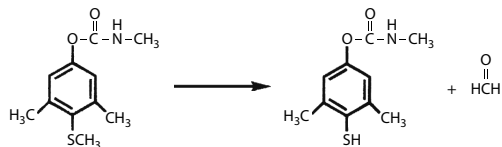


Diazepam also undergoes N-demethylation with the loss of a methyl group as formaldehyde.



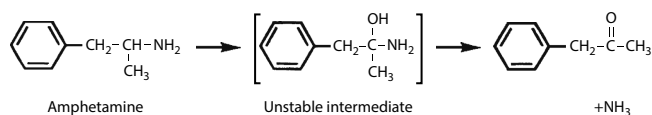
(3) S-Dealkylation

Mesurool, a carbamate insecticide, is S-dealkylated via microsomal cytochrome P450 enzymes.



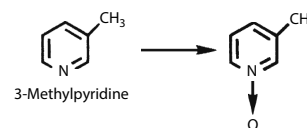
v. Oxidative deamination

Some amines, example, amphetamines, undergo oxidative deamination to yield ammonia and a ketone. This reaction which proceeds through an unstable intermediate is different from monoamine (MAO)-mediated reactions.

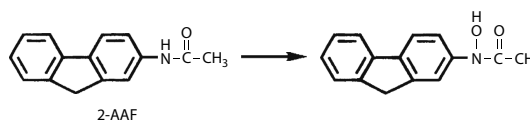


vi. N-Oxidation

Cytochrome P450-mediated N-oxidation may result in N-oxide as in the case of 3-methylpyridine.



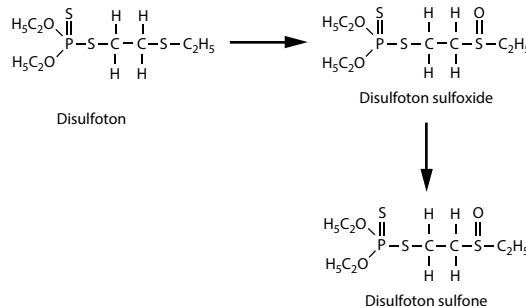
N-Oxidation of other compounds such as 2-acetylaminofluorene (2-AAF) yields a hydroxylamine.



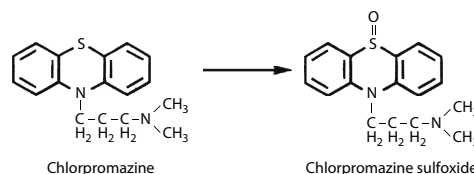
Notably, the hydroxylamine of 2-AAF is considered as the active metabolite that is responsible for the carcinogenicity of 2-AAF.

vii. S-Oxidation

The insecticide disulfoton is oxidized to disulfoton sulfoxide which is subsequently oxidized to disulfoton sulfone.



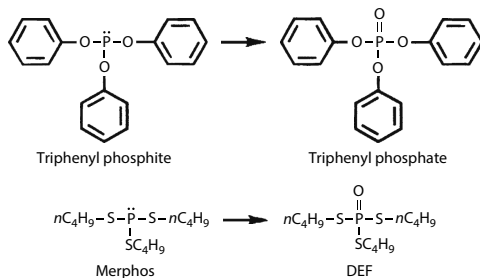
Phenothiazines may also undergo S-oxidation to the sulfoxide derivative.



viii. Phosphorus oxidation

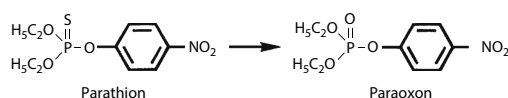
Trisubstituted phosphites are rapidly oxidized to the phosphate or the thiophosphate

as in triphenyl phosphite and merphos, respectively.



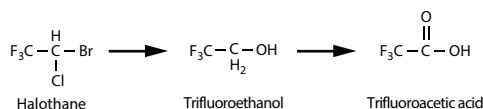
ix. Oxidative desulfuration

Phosphorothioate insecticides are oxidized to phosphates with microsomal cytochrome P450.

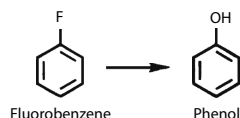


x. Dehalogenation

Halothane, a general anesthetic, undergoes dechlorination and debromination to yield the corresponding alcohol which is subsequently oxidized to an acid.

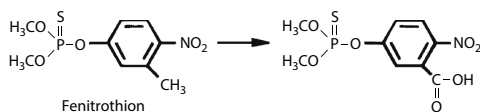


Aromatic halogens are dehalogenated to yield phenols.



xi. Oxidation of aromatic methyl groups

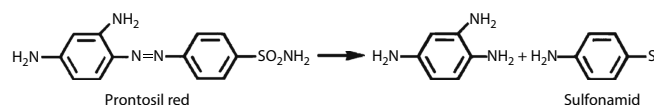
The methyl group of fenitrothion is oxidized to a carboxyl by cytochrome P450.



b. Reduction reactions

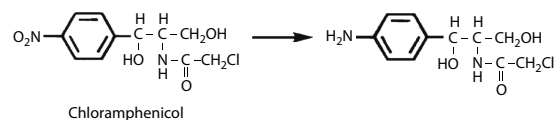
i. Azo reductions

Azo compounds, example, prontosil red, can undergo reductive metabolism catalyzed by cytochrome P450. This reaction resulted in the discovery of sulfonamides.

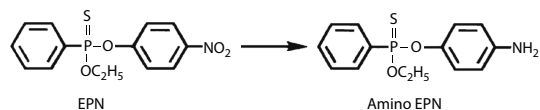


ii. Aromatic nitro reductions

The nitro group in chloramphenicol may be reduced via cytochrome P450.

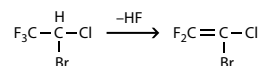


The insecticide EPN undergoes cytochrome P450-catalyzed reduction of its nitro group.



iii. Reductive dehalogenation

Halothane undergoes reductive defluorination via cytochrome P450.



6. Induction of cytochrome P450

Compounds that induce or inhibit the activity of xenobiotic-metabolizing enzymes may play a very important role in the action of xenobiotics on the biological system. These chemicals constitute a large number and include drugs, environmental pollutants, natural products, and pesticides.

a. Enzyme induction

The induction of microsomal enzymes has been demonstrated in many species including humans. Induction may require repeated or chronic exposure to a compound. Exposure may be classified as acute a single exposure, subacute up to 14 days, subchronic up to 90 days, chronic up to life time (18 months in mice) (2 years in rats).

The enzymes induced include cytochrome P450 monooxygenase system glucuronyl transferase UDP-glucose dehydrogenase glutathione transferase esterases microsomal ethanol oxidative system (MEOS) steroid-metabolizing enzymes. There are three types of cytochrome P450 inducers:

- Cytochrome P450 inducers: typical inducer is phenobarbital.
- Cytochrome P448 inducers: typical inducers are dioxin, 3-methyl cholanthrene, and β -naphthoflavone.
- Anabolic steroids, example, testosterone or methyltestosterone, enhance drug metabolism by a mechanism that is different from the other two groups of inducers.

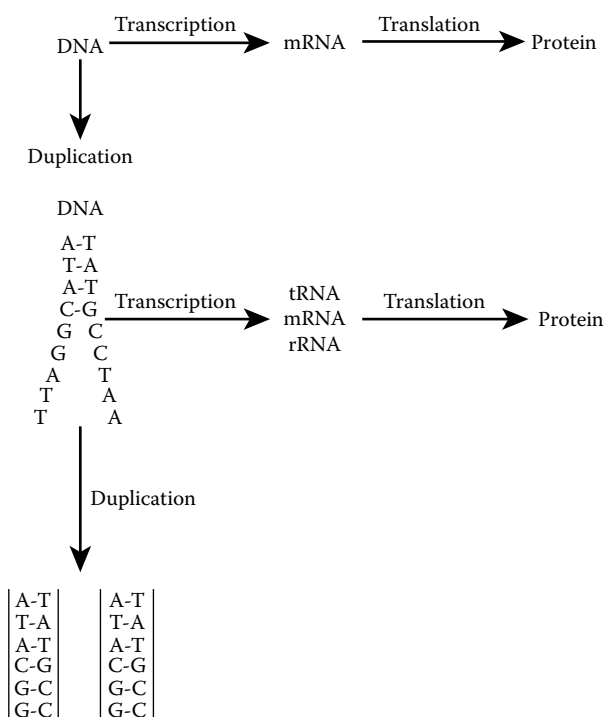
Table 17.9 compares phenobarbital (PB) and 3-methyl cholanthrene (3-MC) induction of cytochrome P450:

- They induce different spectral changes of CO-bound reduced cytochrome P450. PB

increases absorbance at 450 nm. 3-MC or β -naphthoflavone increases absorbance at 448 nm.

- ii. PB causes proliferation of the SER, whereas 3-MC does not.
 - iii. PB induction is general, that is, it induces many enzymes, while 3-MC or β -naphthoflavone are far more specific, that is, induce a few enzymes.
 - iv. PB also increases NADPH cytochrome P450 reductase and RNA synthesis.
- b. Mechanism of cytochrome P450 induction

The genetic code incorporated in DNA molecules consists of pairs of bases arranged in triplets. The bases are the purine bases, adenine (A) and guanine (G), and the pyrimidine bases, thymidine (T) and cytosine (C). The basic mechanism on the transfer of the information encoded in DNA is shown in the following scheme:



Induction:

- i. Is accompanied by an increase in messenger RNA (mRNA) synthesis.
- ii. Is accompanied by an increase in protein synthesis.
- iii. Can be blocked by actinomycin D, an mRNA *synthesis* inhibitor and other inhibitors such as cycloheximide and puromycin.
- iv. The data suggest an *increase* in *de novo* synthesis of enzyme protein takes place.
- v. Increase in protein synthesis may result from a *genetic interaction*, possibly in combination with a “repressor” gene to allow derepression of the operator gene and hence the synthesis of mRNA.

TABLE 17.7

Catalytic Substrates for Major Xenobiotic-Metabolizing CYP Enzymes

Isozyme	Substrate	Reference
CYP1A1	7-Ethoxyresorufin, interferon	Delaporte et al. (1995)
CYP1A2	Phenacetin, caffeine	Bloomer et al. (1995)
CYP1B1	7-Ethoxyresorufin	Crespi et al. (1997)
CYP2A1	Testosterone	
CYP2A6	Coumarin	Nakajima et al. (1996)
CYP2B1	Testosterone	Dehal and Kupfer (1994)
CYP2B6	Coumarin	Ariyoshi et al. (1995)
CYP2C8	Paclitaxel	Sonnichsen et al. (1995)
CYP2C9	Diclofenac	Baldwin et al. (1995)
CYP2C19	(S)-Mephenytoin	Nakamura et al. (1997)
CYP2D6	Bufuralol	Rodrigues and Roberts (1997)
CYP2E1	P-Nitrophenol, chlorzoxazone	Zerilli et al. (1997)
CYP3A4	6 β -Testosterone	Fitzsimmons and Collins (1997)
CYP3A5	6 β -Testosterone	
CYP3A7	6 β -Testosterone	
CYP4A11	Lauric acid	
CYP11B		
CYP17		
CYP19		
CYP21		

TABLE 17.8

Major Cytochrome P450 Gene Families: Substrates, Inducers, and Inhibitors

P450 Gene Family/Subfamily	Characteristic Substrate	Characteristic Inducers	Characteristic Inhibitors
CYP1A2	Acetaminophen Estradiol	Tobacco Char-grilled meats	Cimetidine Amiodarone
CYP2C9	Caffeine Tamoxifen Ibuprofen Fluxetine	Insulin Rifampin Secobarbital	Ticlopidine Fluvastatin Lovastatin Isoniazid
CYP2C19	Diazepam Omeprazole Progesterone	Rednisone Secobarbital	Cimetidine Ketoconazole Omeprazole
CYP2D6	Debrisoquine Ondansertro Amphetamine	Dexamethasone Rifampin	Cimetidine Fluxetine Methadone
CYP2E1	Ethanol Benzene Halothane	Ethanol Isoniazid	Disulfiram Water cress
CYP3A, 5, 7	Cyclosporin Clarithromycin Hydrocortisone Vincristine More drugs	Barbiturates Glucocorticoids Carbamazepine St. John's wort	Cimetidine Clarithromycin Ketoconazole Grapefruit juice More drugs

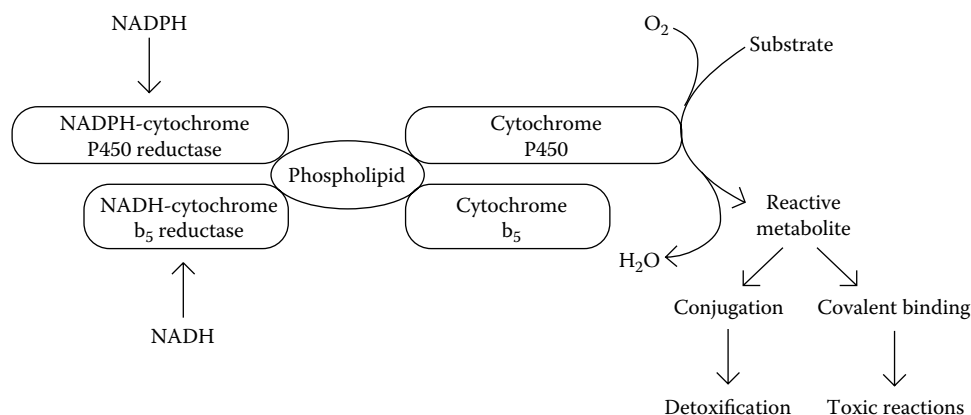


FIGURE 17.1 Schematic presentation of cytochrome P450 components. (Modified after Sipes, I.G. and Gandolfi, A.J., *Casarett and Doull's Toxicology. The Basic Science of Poisons*, 3rd edn., Doull, J., Klassen, C.D., and Andur, M.O., Eds., MacMillian Co., New York, p. 88, 1992.)

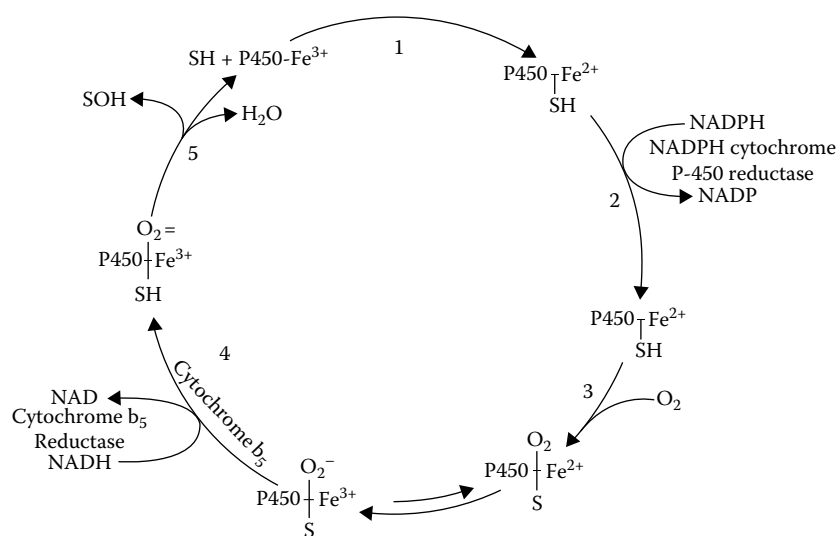


FIGURE 17.2 Schematic mechanism of cytochrome P450-mediated oxidation.

TABLE 17.9
Microsomal Enzyme Induction

Parameter	Phenobarbital	Polycyclic Hydrocarbons
Beginning of induction	8–12 h	3–6 h
Peak induction at	3–5 days	1–2 days
Duration of induction	5–7 days	5–12 days
Enlarged liver	+++	±
Protein synthesis	++++	+
Phospholipid synthesis	+++	–
Liver blood flow	++	–
Biliary flow	++	–
MFO components		
Cytochrome P450	++	–
Cytochrome P448	–	++
NADPH cytochrome <i>c</i> reductase	++	–
Specific enzyme activity		
<i>N</i> -Demethylase	++	–
Aliphatic hydroxylase	++	–
Polycyclic hydrocarbon hydroxylase	+	++
Reductive dehalogenase	++	–
Epoxide hydrolase	++	+
β-Glucuronidase	++	+
Glutathione transferase	+	+

Source: Modified after Sipes, I.G. and Gandolfi, A.J., *Casarett and Doull's Toxicology. The Basic Science of Poisons*, 3rd edn., Doull, J., Klassen, C.D., and Andur, M.O., Eds., MacMillan Co., New York, p. 88, 1992.

Symbols: Large increase, ++++; marked increase, +++; increase, ++; small increase, +; slight increase, ±; no effect, –.

REACTIONS OTHER THAN MICROSOMAL MIXED-FUNCTION OXIDASE

1. Oxidation reactions

There are several enzymes in biological systems that are not related to mixed-function oxidase and can catalyze xenobiotics. These enzymes are microsomal flavin-containing monooxygenase (FMO), alcohol dehydrogenase (ADH), aldehyde dehydrogenase, xanthine oxidase, amine oxidases, aromatases, and alkyldiazine oxidase (Table 17.10).

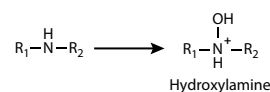
a. Flavin-containing monooxygenase

FMO is present in the microsomes and requires the following cofactors: NADPH, O₂, and reductase. It catalyzes oxygenation reactions of substrates containing N, S, or P atoms (i.e., no C oxidation). FMO catalyzes the oxidation of secondary and tertiary amines, hydrazines, sulfur, and phosphorus compounds.

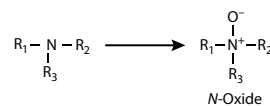
N-Oxidation



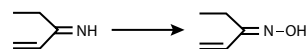
Secondary amines



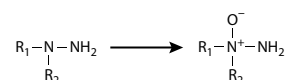
Tertiary amines



Imines



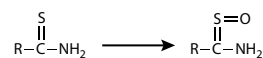
Hydrazines



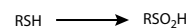
S-Oxidation Thiocarbamide



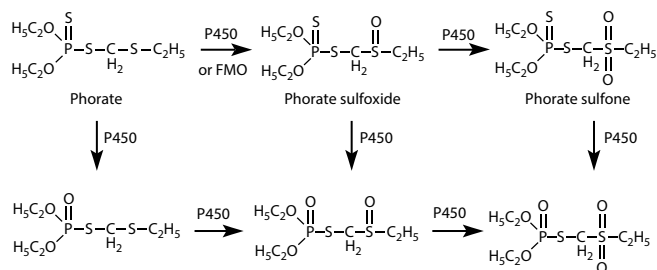
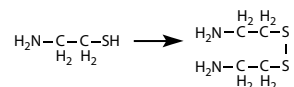
Thioamide



Thiol



Aminothiol

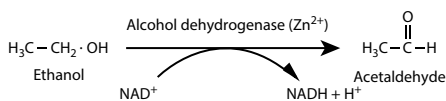


- b. Microsomal ethanol-oxidizing system
Microsomal ethanol-oxidizing system (MEOS) is present in the microsomes of liver and requires

O₂ and NADPH to oxidize ethanol. MEOS has a high K_m for ethanol, ~10 mM, and is responsible for 10%–15% of its metabolism. It is inhibited by carbon monoxide, but unlike cytochrome P450 is not inhibited by SKF 525-A or pyrazole. MEOS activity, hepatic microsomal protein, SER, and cytochrome P450 are increased by prolonged ingestion of ethanol.

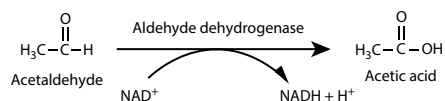
c. Alcohol dehydrogenase

ADH is present in the cytosol (soluble fraction) of liver homogenate. This enzyme has a low K_m , ~1.0 mM, and is responsible for 80%–85% of its oxidation. ADH is not inducible by ethanol or other chemicals.



d. Aldehyde dehydrogenase

Acetaldehyde, the oxidation product of ethanol, undergoes oxidation with acetaldehyde dehydrogenase to form acetic acid. Both enzymes use NAD⁺ as a cofactor.

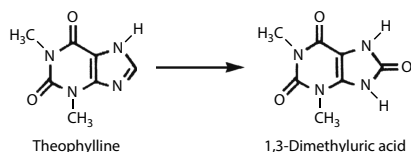


e. Peroxidase–catalase system

This enzymatic system, which is present in peroxisomes in the liver, plays a minor role in the oxidative metabolism of ethanol.

f. Xanthine oxidase

Xanthine oxidase metabolizes xanthine and xanthine-containing drugs such as caffeine, theophylline, and theobromine to the corresponding uric acid derivative.

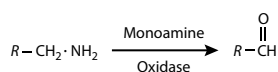


g. Amine oxidases

This class of enzymes is divided into two subclasses: monoamine oxidases and diamine oxidases:

i. Monoamine oxidases

This enzymatic system catalyzes the oxidation of dietary exogenous amines, example, tyramine, to the corresponding aldehyde.



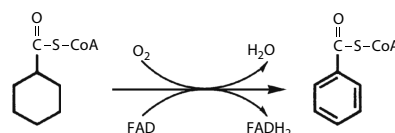
This enzymatic system is found in mitochondria, at nerve endings, and in the liver. This system does not oxidize amphetamines that are metabolized by the microsomal MFO system.

ii. Diamine oxidase

This enzyme mainly oxidizes endogenous substrates.

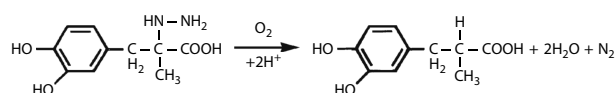
h. Aromatases

This enzymatic system is a mitochondrial enzyme present in the liver and kidney. It requires O₂ and FAD as cofactors. It converts cyclohexane carboxylic acid groups to benzoic acids following the conversion of the acid to the corresponding coenzyme A.



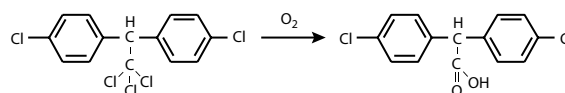
i. Alkylhydrazine oxidase

This enzyme oxidizes alkylhydrazines followed by rearrangement and decomposition of the intermediate, example, the metabolism of carbidopa to 2-methyl-3',4'-dihydroxyphenylpropionic acid.

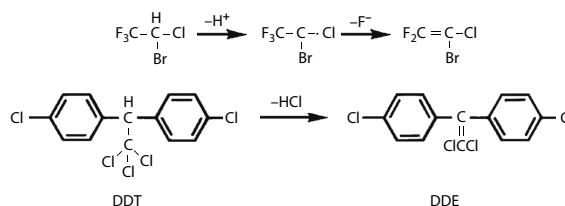


j. Oxidative dehalogenation

This reaction results in the addition of an oxygen in the place of a halogen, such as the conversion of DDT to DDA.



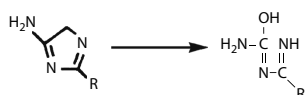
Other examples are the oxidative dehalogenations of halothane and DDT (Abou-Donia and Menzel, 1968).



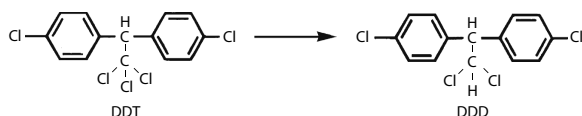
2. Reduction reactions

These reactions require NADPH, but unlike cytochrome P450, they are generally inhibited by oxygen. The following compounds undergo reduction reactions: azo compounds, nitro compounds, epoxides, heterocyclic ring compounds, and halogenated hydrocarbons.

Azo- and nitro-compound reduction may be catalyzed by cytochrome P450, but can also be catalyzed by NADPH cytochrome *c* reductase. Epoxides can be converted back to hydrocarbons. Also, some heterocyclic compounds may undergo ring cleavage by reduction. Such reactions may undergo rearrangement and hydrolysis.



DDT may undergo reductive dechlorination to form DDD.



3. Hydrolysis reactions

Hydrolytic enzymes hydrolyze carboxylic acid esters, amides, carbamates, hydrazides, and phosphoric acid esters.

- a. *Xenobiotic-metabolizing esterases*. Esterases are distributed in the blood and tissues and catalyze the hydrolysis of a variety of esters. These enzymes do not have a well-defined role in the metabolism of either endogenous ester substrates or the metabolism of drugs and other foreign compounds. Most tissues are rich in esterases that have relatively broad structural requirements as far as these substrate specificities and inhibitor sensitivities.

Esterases play a very important role in the development of organophosphorus ester toxicity. This toxicity is dependent upon

- i. The intrinsic toxicity of the organophosphorous ester
- ii. The bioavailability on the concentration of the compound at the site of action. The bioavailability of the organophosphorous compounds at the neurotoxicity target is a function of
 - (1) Route of entry of the chemical
 - (2) Sequestration and subsequent detoxification of organophosphorus compounds by binding or phosphorylation to/by blood proteins, such as albumin, serum ChE (B-esterase), or red blood

cell AChE. Protein-bound organophosphorus esters are secreted into the bile

- (3) Hydrolysis of organophosphorus ester by serum and hepatic A esterases to a water-soluble less toxic metabolites that are excreted in the urine. Hydrolysis is the most important route of the detoxification of organophosphorous esters

Esterases in liver microsomes and cytosol as well as blood and tissues reduce the activity of many esters by acting as scavengers to sequester these esters or hydrolyze them. These enzymes have been classified according to their sensitivity to inhibition by organophosphorus compounds (Aldridge, 1953; Bergmann et al., 1957; Augustinsson, 1961). Xenobiotic-metabolizing esterases include

- i. *Red blood cell*. Acetylcholinesterase (RBC AChE, EC 3.1.1.7) is similar to AChE in nervous system tissues; however, it has no known function in RBC. This enzyme inactivates organophosphorus esters either by binding and sequestering or by hydrolysis.

- ii. *Plasma and tissues*

- (1) *A-Esterases*. These enzymes that are resistant to inhibition by organophosphorus compounds include two subclasses of enzymes:

- (a) Arylesterase or aryl hydrolase (ARE) (EC 3.1.1.2), which is assayed using α -naphthylacetate and aromatic esters as substrates.
- (b) Paraoxonase or phosphoric triester hydrolase (EC 3.1.8.1) that is determined with paraoxon as a substrate. This enzyme is associated with high-density lipoproteins (HDL) in true serum.

- (2) *B-Esterases*. These enzymes are sensitive to inhibition by organophosphates. Two subclasses of enzymes belong to this class:

- (a) Cholinesterase (EC 3.1.1.8), which is also known as plasma or serum cholinesterase, nonspecific esterase, acetylcholine acylhydrolase, cholinesterase II, and

pseudocholinesterase, is an acidic glycoprotein with a molecular weight of 350 kDa and a half-life of 11 days. It is assayed by the use of the substrate butyrylthiocholine.

(b) Carboxylesterase which is also known as aliesterase, Alie (EC 3.1.1.1.), is assayed using *p*-nitrophenyl acetate.

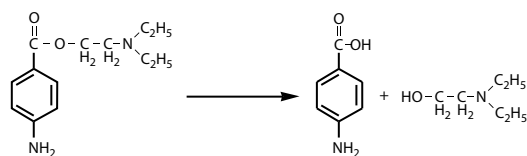
(3) *C-Esterase*, which is also known as acetylcholinesterase (EC 3.1.1.6), is resistant to inhibition by diisopropylfluorophosphate (DFP) but activated by *p*-chloromercuric benzoate and phenylmercuric acetate.

A-esterases and B-esterases including ChE activities vary widely within healthy population and are influenced by both genetic and environmental factors as well as diseased states. Variation in the activities of these enzymes may contribute to interindividual differences in susceptibility to organophosphorous compound toxicity.

iii. *Lymphocytes and tissues.* Neurotoxic esterase or neurotoxicity target esterase (NTE), which is present in lymphocytes and most tissues, is assayed using phenylvalerate as a substrate (Johnson, 1977). It is a good biomarker for organophosphorus ester-induced delayed neurotoxicity (OPIDN) which correlates well with its inhibition and aging. Organophosphorus compounds capable of producing OPIDN results in at least 70% inhibition of NTE. Like other nonspecific esterases, neither its natural biochemical nor physiological function is known.

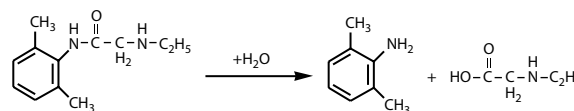
b. Carboxylic acid ester hydrolysis

Hydrolysis of these esters is carried out by esterases present in plasma, example, pseudocholinesterases, or in the liver to produce carboxylic acid and alcohol.



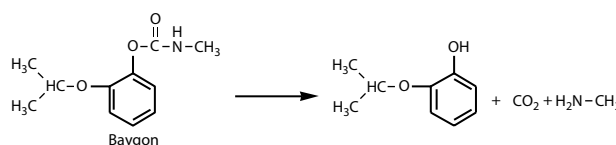
c. Amide hydrolysis

This reaction is catalyzed by amidases to yield an amine and an acid.



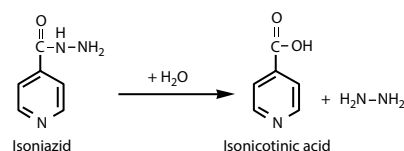
d. Carbamates

Aromatic carbamates are hydrolyzed to yield phenols.



e. Hydrazides

Isoniazid is hydrolyzed to isonicotinic acid and hydrazine.



4. Hydration reactions

Epoxide hydratase catalyzes the hydration of epoxides. This reaction involves the addition of a water molecule to produce the dihydrodiol.

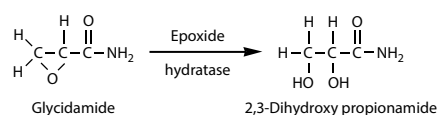


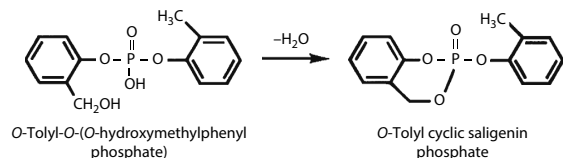
TABLE 17.10

Cytochrome P450 and FMO

Parameter	P450	FMO
Location	Microsomes	Microsomes
Cofactors	NADPH and O ₂	NADPH, O ₂ , reductase
Inducers	Phenobarbital, 3-methylcholanthrene, ethanol	None
Substrates	Numerous	Few
Reactions	Oxidation, reduction	Oxidation
Oxidation	C, N, S, P	N, S, P

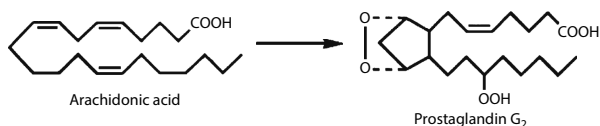
OTHER PHASE I REACTIONS

Cyclization



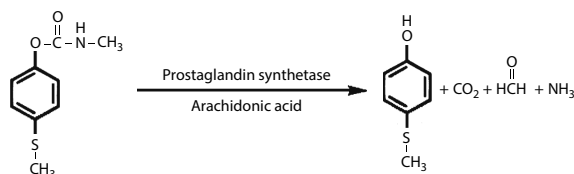
PROSTAGLANDIN ENDOPEROXIDE SYNTHETASE

The prostaglandin endoperoxide synthetase system involves two enzymes: fatty acid cyclooxygenase and hydroperoxidase. Fatty acid cyclooxygenase catalyzes bis-dioxygenation to produce the hydroperoxy endoperoxide PGG₂.



On the other hand, hydroxyperoxidase catalyzes the reduction of PGG₂ to PGH₂.

This enzymatic system metabolizes *N*-methyl carbamates that have a pair of electrons available for the last step of the reaction in the presence of arachidonic acid, resulting in demethylation. Neither S- nor O-demethylation takes place.



ENDOGENOUS METABOLISM BY PHASE I ENZYMES

Phase I enzymes carry out the metabolism of many endogenous substrates. Table 17.11 summarizes these reactions.

TABLE 17.11

Endogenous Metabolism by Phase I Enzymes

Phase I Enzyme	Endogenous Substrate
Acetylcholinesterase	Acetylcholine
Diamine oxidase	Cadaverine
	Histamine
	Putrescine
Hydroxysteroid oxidoreductase	Steroids
Mixed-function oxidase	Fatty acids
	Leukotrienes
	Prostaglandins
	Steroids
	Sterols
	Thyroid hormones
	Vitamins
Monoamine oxidase	Monoamine neurotransmitters
Reductases	Steroids
Xanthine oxidase	Xanthines

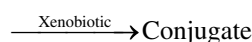
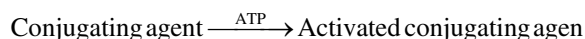
XENOBIOTIC-METABOLIZING REACTIONS: PHASE II

It is common that a xenobiotic is subjected to several competing reactions simultaneously. Also, some metabolic reactions proceed sequentially and Phase I reactions, that is, oxidation, reduction, and hydrolysis, are followed by Phase II or conjugation reactions of products.

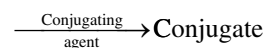
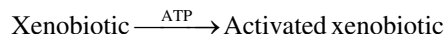
Phase II reactions involve the addition of a xenobiotic to endogenous groups that are generally polar and readily available *in vivo* (Table 17.12). This process renders the whole molecule more acidic, more polar, and less lipid-soluble, thus facilitating elimination from the body. The conjugates formed are usually less toxic than their parent compounds; therefore, Phase II is considered a detoxification mechanism.

Conjugation requires (1) ATP as a source of energy, (2) coenzymes, and (3) transferases. Conjugation reactions and transferases are listed in Table 17.13. The conjugation usually proceeds in two steps: (1) extramicrosomal synthesis of an acylcoenzyme and (2) the transfer of the acetyl moiety to the acylcone, which in some but not all cases, is localized in microsomes.

All conjugation reactions require activated nucleotides as activated intermediates. There are two conjugation mechanisms: (1) activated conjugating agents



and (2) xenobiotic or its metabolite activated



Tissue and subcellular localization of conjugation reactions are listed in Table 17.14.

TABLE 17.12

Conjugation Reactions of Phase II

Conjugating Chemical	Product
Glucuronic acid	Glucuronide
Sulfuric acid	Ethereal sulfate
Glycine	Hippuric acid
Glutamine ^a	Glutamine conjugate
Glutathione	Mercapturic acid
Methyl group	Methyl ether
Acetic acid	Acetyl ester
Thio group	Thiocyanate
Glucoside conjugation ^b	Glucoside
Ornithine conjugation ^c	Ornithine conjugate

^a Occurs only in man, apes, and new and old world monkeys.

^b Occurs in insects.

^c Occurs in certain birds.

TABLE 17.13

Conjugation Reactions, Transferase Enzymes, and Functional Groups of Phase II

Reaction	Transferase	Functional Group
Glucuronidation	UDP-Glucuronyltransferase	–OH –COOH NH ₂ –SH
Glycosidation	UDP-Glycosyltransferase	–OH –COOH –SH
Sulfation	Sulfotransferase	–OH –NH ₂ –SO ₂ NH ₂
Methylation	Methyltransferase	–OH –NH ₂
Acetylation	Acetyltransferase	–OH –NH ₂ –SO ₂ NH ₂
Amino acid conjugation		–COOH
Glutathione conjugation	Glutathione-S-transferase	Epoxide Organic halide
Fatty acid conjugation		–OH

TABLE 17.14

Tissue and Subcellular Localization of Conjugation Reactions of Phase II

Conjugation Reaction	Intermediate Nucleotide	Localization	
		Tissue	Subcellular
a. Activated conjugation			
Glucuronidation	Uridine diphosphate, glucuronic acid	Liver, most tissue	Microsomes
Ethereal sulfation	Phosphadenosine, phosphosulfate	Liver, kidney, intestine	Supernatant
Methylation	S-Adenosylmethionine	Liver, many tissue	Microsomes, supernatant
Acetylation	Acetyl CoA	Liver, other tissue	Supernatant
b. Activated xenobiotic			
Hippuric acid	Aryl CoA	Liver, kidney	Mitochondria
Glutamine synthesis	Phenacetyl CoA	Liver, kidney	Mitochondria

CONJUGATION WITH SUGARS

Conjugation with α -D-glucuronic acid to produce glucuronides is the most common sugar conjugation reaction. Other conjugations with sugars include conjugation with glucose, xylose, and ribose.

1. Glucuronidation

a. Mechanisms of the reaction

- i. Glucuronide formation is one of the most common routes of drug metabolism because of the availability of glucose.

- ii. The reaction involves the condensation of D-glucuronic acid.

- iii. This reaction requires the activation of glucuronic acid by the synthesis of uridine diphosphate glucuronic acid (UDPGA, "H" is α).

- iv. Glucuronidation involves nucleophilic attack (SN_2) by the oxygen, sulfur, or nitrogen atom at C-1 carbon atom of the glucuronic acid moiety. Therefore, the H atom of glucuronic acid is inverted to a

β -configuration when complexed with the xenobiotic compound.

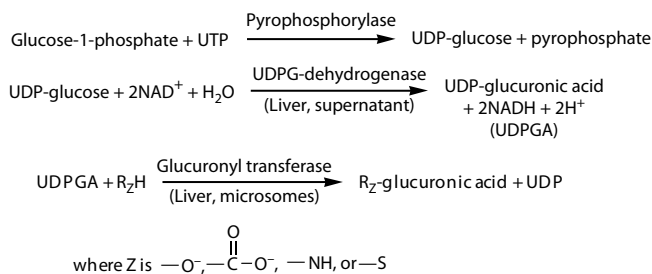
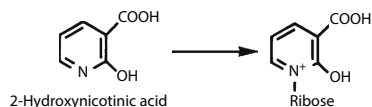


Table 17.15 lists various types of conjugation reactions.

b. Other sugars

Unlike mammals, conjugation with glucose is most prevalent in insects. This reaction proceeds in an analogous fashion to glucuronide formation but UDP-glucose is used instead of UDPGA, resulting in glucoside formation. Similarly, *O*-, *N*-, and *S*-glucosides may be formed. These reactions are also formed in plants.

In some instances, UDP-xylose or UDP-ribose can be used to form xylosides or ribosides. An example of *N*-riboside formation is shown as follows:

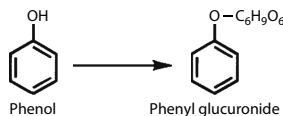


2. Substrates for the reaction

a. O-Conjugation

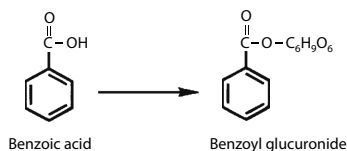
i. Ether type

Alcohols and phenols form “ether-type” glucuronides.



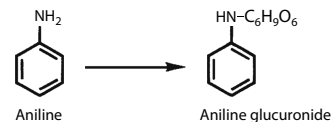
ii. Ester type

Aromatic and some aliphatic carboxylic acids form “ester-type” glucuronides.

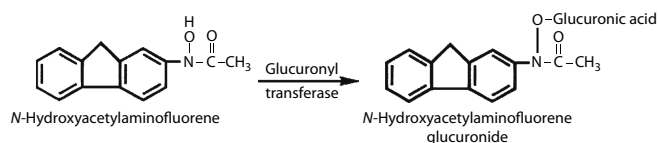


b. N-Glucuronide

Amines, especially aromatic compounds, form *N*-glucuronides.

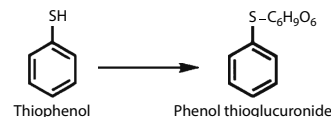


Although conjugation generally decreases biological activity, including toxicity, occasionally there are exceptions. The *N*-hydroxyglucuronide is a more potent carcinogen than the parent compound acetaminofluorene.



c. S-Glucuronide

Certain thiol compounds form *S*-glucuronides.



In addition, normally occurring substrates, such as steroids, thyroxine, and bilirubin, also conjugate with glucuronic acid.

3. Glucuronide formation in various species

- Glucuronidation takes place in most mammalian species with the exception of the cat.
- The cat can synthesize UDPGA, but it lacks glucuronyl transferase.
- Fish lack glucuronidation because they are deficient in UDPGA.
- In rats, males produce more glucuronides than females. This sex difference does not hold for humans, however.

4. Factors affecting glucuronide formation

- Inducers of drug metabolism increase the activity of glucuronyl transferase.
- MFO inhibitors, such as SKF-525A, inhibit glucuronyl transferase activity *in vivo*.
- Glucuronide formation is diminished during pregnancy. This may be explained by the increased level of progesterone and pregnane-diol which inhibit transferase activity.
- Patients with liver damage may have impaired glucuronide conjugation.
- The newborn in humans and most species, except in the rat, has very low levels of glucuronyl transferase. In infants, the failure to

conjugate chloramphenicol and its metabolites to nontoxic glucuronides results in the characteristic “Gray Baby” syndrome, characterized by cyanosis, cardiovascular toxicity, and death.

5. Excretion of glucuronides

a. Urinary excretion

- i. Glomerular filtration—high-molecular-weight conjugates such as glucuronides of androsterone and pregnanediol are eliminated by glomerular filtration alone.
- ii. Passive tubular diffusion—low-molecular-weight conjugates, that is, phenols, are excreted by passive tubular diffusion.
- iii. Active secretion—this is a carrier-mediated active transport process. Medium-molecular-weight glucuronides are frequently excreted by tubular secretion.

b. Bile excretion

- i. Biliary excretion in the rat is preferred for compounds with molecular weight of 325,

having one or more aromatic rings, and one or more hydroxyl groups. Thus, glucuronides of bilirubin, thyroxine, pregnanediol, morphine, and chloramphenicol are mostly excreted into the bile.

- ii. Glucuronides excreted via the bile in the gut (duodenum) may be hydrolyzed by β -glucuronidase (lysosomes). The free chemical may undergo absorption, transport to the liver, reconjugation, and reexcretion (enterohepatic circulation).

6. Methods to study glucuronidation

- a. It is possible to isolate glucuronides by precipitation from solutions followed by crystallization.
- b. It is more customary to identify glucuronides by the use of β -glucuronidase enzyme. In these studies, radiolabeled xenobiotics are extracted with organic solvents followed by hydrolysis by incubation with β -glucuronidase. Glucuronides are then extracted with organic solvents.

TABLE 17.15
Types of Glucuronidation Reactions

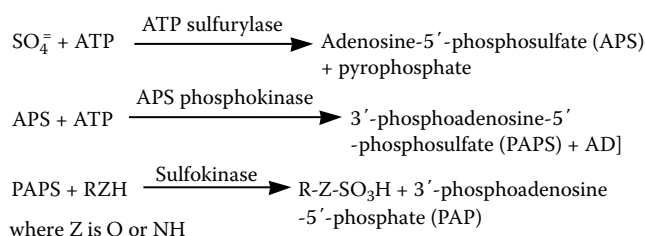
Functional Group	Type of Chemical	Structure	Example
Hydroxyl	Phenol	ArOH	Morphine
	Enol	$-\text{CH}=\text{COH}$	4-Hydroxycoumarin
	Primary alcohol	$-\text{CH}_2\text{OH}$	Chloramphenicol
	Secondary alcohol	$\begin{array}{c} \diagup \\ \text{CH} \\ \diagdown \end{array} - \text{OH}$	<i>sec</i> -Butanol
	Tertiary alcohol	$\begin{array}{c} \\ -\text{C}-\text{OH} \\ \end{array}$	<i>tert</i> -Butanol
	Hydroxylamine	$\begin{array}{c} \text{H} \\ \\ \text{H}-\text{O}-\text{N}-\text{H} \end{array}$	<i>N</i> -Hydroxy- <i>N</i> -2-fluorenylacetamide
Carboxyl	Aromatic acids	$\begin{array}{c} \text{O} \\ \\ \text{Ar}-\text{C}-\text{OH} \end{array}$	Salicylic acid
	Aliphatic acids	$\begin{array}{c} \text{O} \\ \\ -\text{CH}_2-\text{C}-\text{OH} \end{array}$	Indomethacin
Amino	Aromatic	ArNH ₂	4,4'-Diaminodiphenylsulfone
	Carbamate	$\begin{array}{c} \text{O} \\ \\ -\text{OC}-\text{NH}_2 \end{array}$	Meprobamate
Imino	Sulfonimide	$\begin{array}{c} \text{O} \\ \\ -\text{S}-\text{NH}- \\ \\ \text{O} \end{array}$	Sulfadimethoxine
	Heterocyclic	$\begin{array}{c} \diagup \\ \text{NH} \\ \diagdown \end{array}$	Sulfisoxazole
Sulfhydryl	Thiol	$-\text{SH}$	2-Mercaptobenzothiazole
	Carbodithioic	$\begin{array}{c} \text{S} \\ \\ -\text{C}-\text{SH} \end{array}$	Diethyldithiocarbamic acid

SULFATION

Conjugation by sulfate is a very efficient pathway for eliminating xenobiotics through urine because the sulfate conjugates are completely ionized and highly water-soluble. The major compounds that undergo sulfation reactions are alcohols, phenols, and arylamines. This reaction is catalyzed by the enzyme sulfotransferase (Table 17.16).

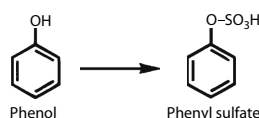
1. Mechanisms of the reaction

The coenzyme participating in sulfuric acid conjugation is 3-phosphoadenosine-5-phosphosulfate (PAPS). Sulfate conjugation reactions involve ATP and take place in the soluble fraction of cells.

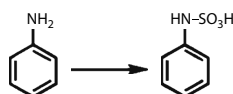


2. Substrates for the reaction

- a. Etheral sulfates—phenol and alcohols form “etheral sulfates.”



- b. Sulfamates—aromatic amines form “N-sulfates” or sulfamates.



Endogenous sulfate conjugates include heparin, tyrosine sulfate, various sulfolipids, the sulfates of epinephrine, norepinephrine, progesterone, and estrone.

3. Factors affecting sulfate conjugation

- The total pool of sulfate is usually quite limited and can be readily exhausted; thus with increasing doses of a drug, conjugation with sulfate may become a zero-order reaction.
- For this reason, conjugation with glucuronic acid is more predominant over that of sulfate.
- Most species, including man, are able to make sulfate derivatives, although to less extent in the pig and fish.
- At birth, relatively little sulfokinase activity is present in many animals similar to that with glucuronyl transferase.
- Sulfatases which can cleave the sulfate group from their derivatives are present in most species (liposomes).

TABLE 17.16
Sulfation Reactions

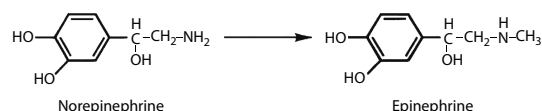
Compound	Enzyme	Tissue
Dimetranidazol	Alcohol sulfotransferase	Liver
Isoprenaline	Phenol sulfotransferase	Liver, kidney, gut
Oestrone	Steroid sulfotransferase	Liver
Paracetamol	Arylamine sulfotransferase	Liver

4. Methods for studying sulfate conjugation

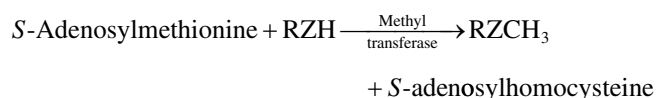
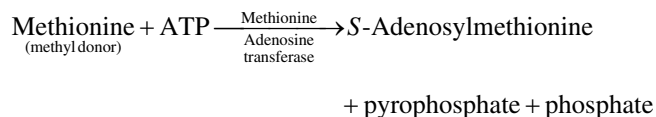
- Precipitation
- Hydrolysis with sulfatases

METHYLATION

Methylation represents a relatively minor metabolic pathway for drugs. It differs from other conjugation reactions in that the products formed occasionally have extensive biological activity, for example, epinephrine (Table 17.17).



1. Mechanisms of the reaction



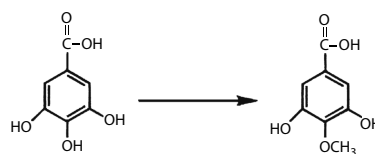
Methyl transferases are localized in liver microsomes.

2. Substrates for the reaction

- a. N-Methylation



- b. O-Methylation



- c. S-Methylation

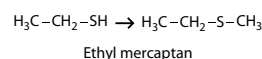


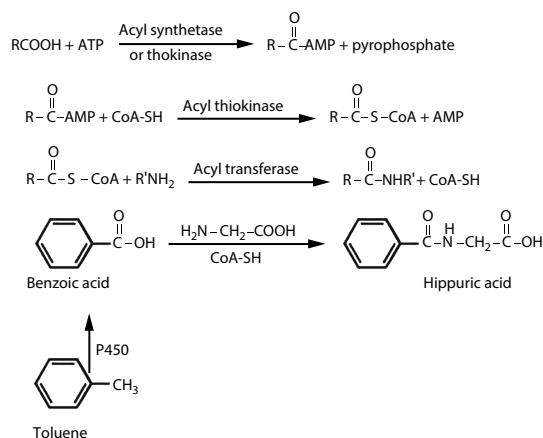
TABLE 17.17
Methylation Reactions

Compound	Enzyme	Tissue
<i>N</i> -Acetylserotonin	Hydroxyindole <i>O</i> -methyltransferase	Pineal gland
Catechols	Catechol <i>O</i> -methyltransferase	Liver, kidney, skin, nervous tissue
Histamine	Imidazole <i>N</i> -methyltransferase	Liver
Noradrenaline	Phenylethanolamine <i>N</i> -methyltransferase	Adrenals

AMIDE SYNTHESIS

This reaction takes place in the mitochondria of liver and kidney cells. This reaction involves the condensation of an acid with an amine to form an amide. Two types of reactions will be considered:

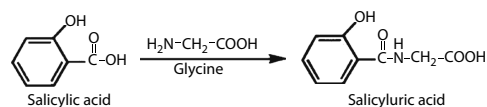
1. Conjugation of carboxylic acid-containing drugs with endogenous amines (usually amino acids, example, glycine). The coenzyme participating in this reaction is acetyl coenzyme A.



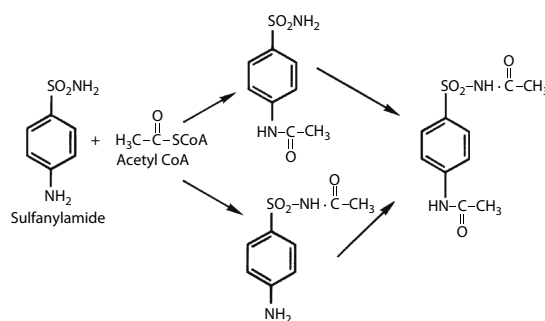
a. Glycine

- (1) The formation of hippuric acid from benzoic acid and endogenous glycine was first demonstrated in the horse, and the name "hippuric acid" was chosen from the Greek word "hippos" for horse.
- (2) For other acids, the ending "uric" still applies to the metabolite even though

it has no relation to uric acid; thus, salicyluric acid is the glycine conjugate of salicylic acid.



- (3) The pool of endogenous glycine is limited, and hippuric acid formation may follow zero-order kinetics.
 - (4) Glycine conjugation may be impaired in certain cases of liver diseases, and hippuric acid formation following benzoic acid ingestion has been used as a test for liver function.
 - (5) In the newborn and in the elderly, glycine is less available and hippuric acid formation is reduced.
- b. Other amino acids
- (1) Glutamine—conjugates acid drugs in primates and certain primates
 - (2) Ornithine—conjugation with ornithine takes place in reptiles and birds
2. Conjugation of amine drugs with endogenous carboxylic acids, example, acetic acid
 - a. This takes place with aromatic primary amines, sulfonamides, and hydrazines.



- b. Phenols, alcohols, or thiols do not form acetyl derivatives.
- c. The dog, unlike most mammalian species, is a poor acetylator.
- d. Acetylation of isoniazid is genetically determined in humans. Slow acetylators have less *N*-acetyltransferase in their liver than rapid acetylators (inactivators).

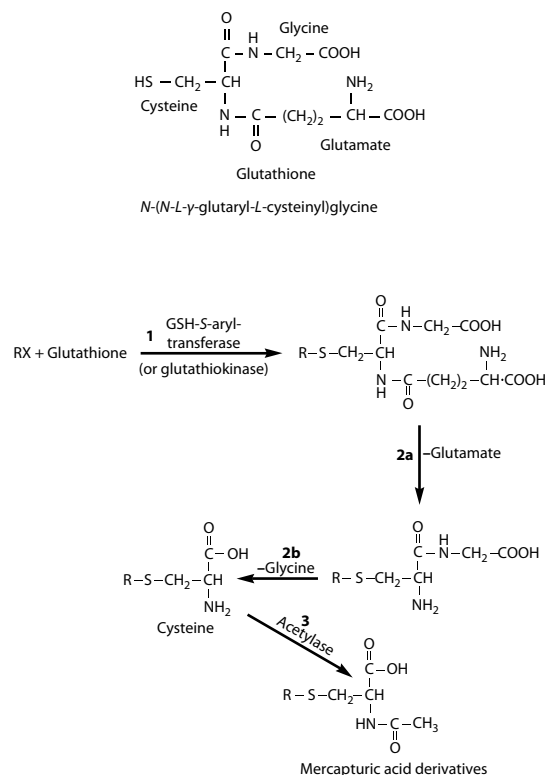
- e. Humans lack the ability to deacetylate acetylated aromatic amines, and the apparent rate of acetylation is relatively great.

GLUTATHIONE CONJUGATION (MERCAPTURIC ACID SYNTHESIS)

This reaction results in *N*-acetylcysteine (mercapturic acid conjugates) as follows:

1. An initial conjugation with glutathione (glutathione-*S*-transferase)
2. Cleavage of the glutamyl and glycyl residues
3. Acetylation of the glycine moiety

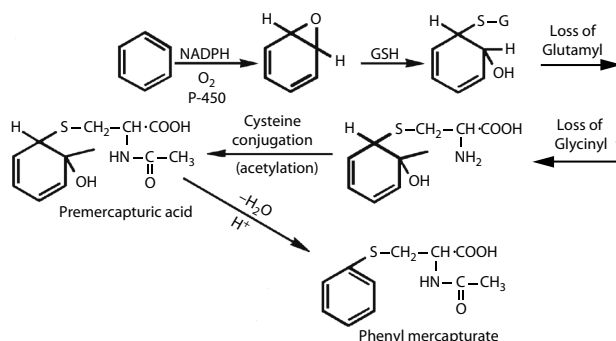
The first step is catalyzed by glutathione-*S*-transferase that is primarily present in the soluble (cytosol) fraction of the cell, but is also found in the microsomes.



Glutathione conjugation is a detoxification reaction since it masks reactive electrophiles.

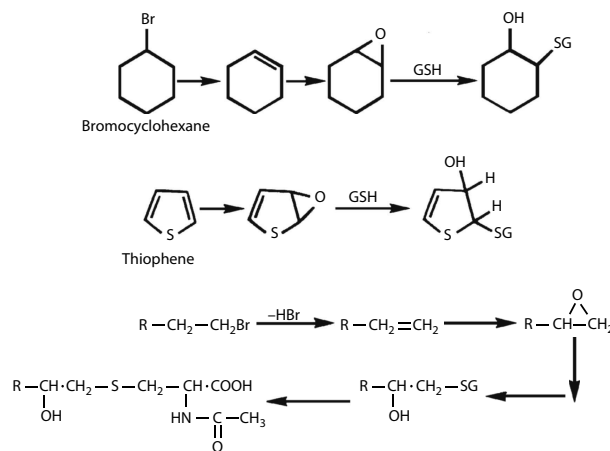
Examples:

1. Conjugation of benzene epoxide with glutathione and formation of phenyl mercapturate

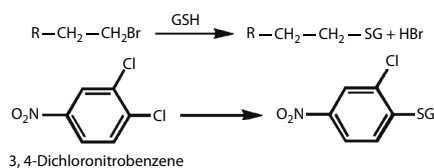


Xenobiotics subject to this reaction frequently contain an active halogen or a nitro group.

2. Conjugation of various epoxides with glutathione



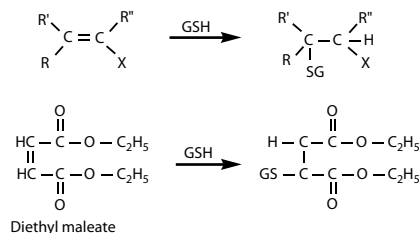
3. Displacement of aliphatic and aromatic halogens by glutathione



4. Unsaturated aliphatic compounds with suitable electron-withdrawing groups.

Chemicals such as diethyl maleate may react directly with glutathione *without* undergoing metabolic

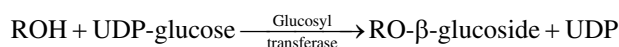
activation. Diethyl maleate may be used to deplete hepatic glutathione *in vivo* experimentally.



OTHER CONJUGATION REACTIONS

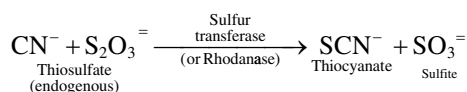
1. Glucoside conjugation

This reaction occurs in insects and plants where it replaces glucuronide conjugation. Ester and ether glucosides are formed:



Mammals do not synthesize glucosides because they lack glucosyl transferase.

2. Thiocyanates



- Free cyanides or cyanides formed from nitriles or oximes from thiocyanates.
- Sulfur transferase is found mainly in liver mitochondria.

PHASE II METABOLISM OF ENDOGENOUS COMPOUNDS

Endogenous compounds undergo conjugation via Phase II metabolic pathways. Examples of these reactions are listed in Table 17.18.

TABLE 17.18

Phase II Metabolism of Endogenous Compounds

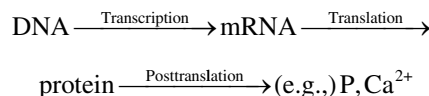
Conjugation Reaction	Substrates
Acetylation	Serotonin
Amino acid conjugation	Bile acid
Glucuronidation	Steroids
	Thyroxine
	Bilirubin
	Catecholamines
Glutathione conjugation	Arachidonic acid metabolites (leukotrienes)
Methylation	Biogenic amines
Sulfation	Carbohydrates
	Steroids

TOXIC RESPONSES TO XENOBIOTICS

Xenobiotics may exert their effects (pharmacological or toxic) at various levels, molecular, subcellular, cellular, tissue, and at the organism level.

MOLECULAR CHANGES

- Interactions with nucleic acids, example, DNA, RNA, leading to irreversible conformational changes, and causing mutations or carcinogenesis:



- Interactions with proteins, leading to denaturation, precipitation, allosteric effects (change in reactivity), or enzyme inhibition.

SUBCELLULAR CHANGES

- Action on the permeability of cell membranes and disturbances of energy metabolism (ATPase), example, free radicals.
- Decrease of the stability of lysosomal membranes resulting in the release of hydrolases and leading to the disruption of the cell.

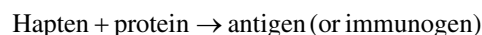
CELLULAR CHANGES

Action at the cellular level causes deranged reproduction, differentiation, and maturation resulting in teratogenesis.

ALLERGIC OR SENSITIZATION REACTIONS

Allergic reactions result from previous sensitization to a chemical. To produce an allergic reaction,

- A chemical functions as a hapten:



- Immune cells produce antibodies against the antigen:



- Subsequent exposure to the chemical yields an antigen, resulting in antigen-antibody interaction. This produces typical manifestations of allergy, example, dermatitis, itching, watery eyes, or bronchiolar constriction.

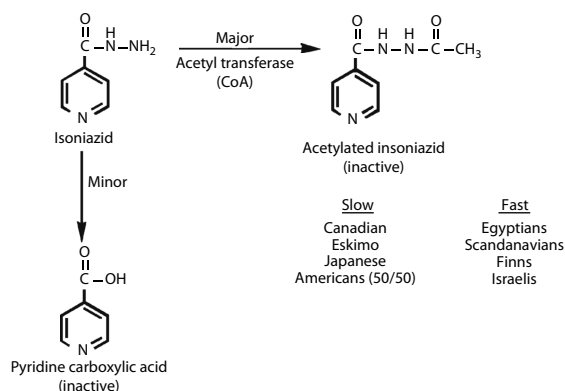
IDIOSYNCRASY

Idiosyncratic drug reactions (IDRs) are defined as genetically determined abnormal reactivity to drugs and also referred to as type B (bizarre) adverse drug reactions (ADRs, Zhang et al., 2011). ADRs occur only in a small proportion of patients who take drugs and are not related to the therapeutic effects of the drugs. ADRs are a major cause of patient morbidity and mortality. Although IDRs make up only about 5% of ADRs, they can be very severe, example, idiosyncratic drug-induced liver injury was responsible for nearly 13% of acute liver failure in the United States from 1997 to 2001 (Lee, 2003). A major characteristic of IDRs is the delay of a week or more in onset of adverse effects (Uetrecht, 2008).

Examples include reactions to isoniazid and succinylcholine.

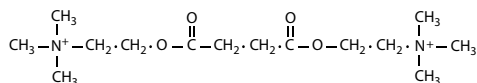
1. Isoniazid

Isoniazid is used in chemotherapy for tuberculosis.



Both fast and slow acetylators have the same enzyme. Slow acetylators have less of the enzyme: less synthesis and/or more degradation of the enzyme.

2. Succinylcholine



1. Used in anesthesia as a neuromuscular-blocking agent—depolarizing type
2. Used to produce muscle relaxation during surgery
3. Used because it has short duration due to its hydrolysis by plasma pseudocholine esterase or butyrylcholinesterase (BuChE)
4. Some individuals have “atypical” BuChE, which hydrolyzes succinylcholine at a slow rate resulting in a prolonged muscular relaxation and apnea (asphyxia) for several hours
5. The “atypical” enzyme has 100-fold *less* affinity to succinylcholine

TRANSPORTERS

Active transport of drugs, toxicants, and their metabolites plays an important role in therapeutic and toxic actions. Several transporters have been identified in the liver, kidney,

intestine, and lung that transport various substances including sugars, amino acids, and peptides as well as drugs and pesticides.

P-GLYCOPROTEIN

P-gp, an adenosine triphosphate (ATP)-dependent chemical transporter, is involved in the development of multidrug resistance (MDR) in cancer chemotherapy and pesticide resistance. The presence of this protein, a membrane of the large ATP-binding cassette (ABC) transporter family, decreases intracellular accumulation of cytotoxic drugs, thereby increasing tumor cell despite the presence of otherwise toxic drug cellular levels. P-gp acts as a barrier to absorption of xenobiotics and assists in their removal. Thus, P-gp may diminish their absorption from the intestine and reduce oral bioavailability.

1. MDR gene family

P-gps belong to a gene family known as the *MDR* genes. Human and other primates have two members of the gene family, *MDR1* and *MDR2* (sometimes known as *MDR3*). On the other hand, mice, hamsters, and rats have three members, that is, *mdr1a*, *mdr1b*, and *mdr2*. These genes encode drugs or phospholipid transporters listed in Table 17.19.

2. Structure and function of P-gp

MDR1 gene is 100 kb on chromosome 7, with 28 exons that are spliced into 4.5 kb mRNA. P-gp consists of 1280 amino acids within the amino and carboxy halves of the protein each have six trans-membrane domains and an ATP-binding region. Table 17.20 lists compounds that interact with P-gp. Increased expression of P-gp is associated with multidrug-resistance phenomenon in which cells become cross resistant to structurally related chemicals. Multidrug-resistant phenotype confers into drug-sensitive cells by transfecting DNA isolated from drug-resistant cells.

3. Tissue distribution of P-gp

P-gp is present in the epithelial cells lining the luminal surface of tissues associated with excretory or barrier functions such as hepatic bile canalicular membrane, renal proximal tubule, villus-tip enterocyte in the small intestine, and the endothelial cells making up the BBB and blood–testes barrier. The extent of mRNA P-gp in tissues is listed in Table 17.21.

4. Significance of P-gp in pharmacokinetics of drugs

P-gp facilitates the intestinal absorption of drugs and their excretion into bile. Coadministration of the P-gp inhibitors PSC 833 or CsA with paclitaxel results in a 10-fold increase in AUC. These results suggest the combined contribution of CYP3A inhibition in the intestine and liver in decreasing oral bioavailability of substrate drugs. P-gp also

decreases the oral bioavailability of the HIV protease inhibitors indinavir, nelfinavir, and saquinavir, suggesting more effective therapeutic action of these drugs would result from coadministration with P-gp inhibitors.

Recently, a significant amount of drug metabolism has been shown to take place in the intestine that is mediated by CYP3A. Since >50% of the clinically important drugs are metabolized by CYP3A, its presence in the intestine suggests an important role in oral drug bioavailability. A striking overlap between the substrates for P-gp and CYP3A has been established.

Compounds used to modify or reverse MDR include calcium channel blockers (verpamil, nifedipine), immunosuppressives (CsA, FK506), and antiarrhythmic drugs (quinidine and amiodarone). Table 17.22 shows a partial list of MDR reversal agents.

These compounds reverse P-gp by various mechanisms. Some limitation, such as CsA, a substrate itself, acts as a competitive inhibitor. Mechanisms with other agents are still unknown. Competition may result from binding at the same site. In summary, P-gp, a xenobiotic transporter presents in the kidney, liver, intestine, blood, brain, and other epithelial tissues, plays a significant role in interactions between substrate chemicals and their subsequent bioavailability.

5. P-gp as an absorption barrier

Tumor necrosis factor α (TNF- α) is a cytokine with antitumor activity against several cellular models. TNF- α -induced apoptosis seems to be mediated by a signaling pathway termed sphingomyelin-ceramide pathway, which consists of the hydrolysis of sphingomyelin and the production of its breakdown product ceramide. The KGla cells, which are inherently resistant to TNF- α and do not produce ceramide upon cytokine stimulation, can be sensitized by the use of the P-gp inhibitor PSC833. Coincubation with 1 μ M of this cyclosporin derivative restored the apoptotic potential of 10 ng/mL TNF- α . This effect was associated with the restoration of ceramide generation (315%) and activation of neutral, but not acid sphingomyelinase activity (143%). Furthermore, it has been demonstrated that treatment of KGla cells with 1 μ M PSC833 led to a threefold increase in inner plasma membrane sphingomyelin content and basal neutral sphingomyelinase activity. These results support the hypothesis whereby resistance to TNF- α -mediated apoptosis of certain leukemic cells is linked to the disposability of the sphingomyelin pool. These data also suggest a role for P-gp in sphingomyelin transverse plasma membrane asymmetry.

Among the compounds endowed with the capacity to reverse the P-gp-mediated MDR of cancer cells, a powerful agent was found to be the cyclosporin D derivative SDZ PSC 833. The brain penetration of SDZ PSC 833 and its effect on the BBB permeability of an anticancer agent vincristine were assessed. At lower doses of SDZ PSC 833 the brain penetration, defined as the brain-to-blood partition coefficient (K_p), was very low in spite of the high lipophilicity of this compound. At higher doses, however, the brain penetration of SDZ PSC 833 proved to be linear in the dose range studied; these results demonstrated a dose-dependent brain passage of SDZ PSC 833. The brain passage of cyclosporin A was also found to be dose-dependent. On the other hand, 10 times higher doses of cyclosporin A were required to obtain the same K_p values recorded for SDZ PSC 833. Moreover, the coadministration of SDZ PSC 833 increased the brain penetration of cyclosporin A, whereas the latter did not modify that of SDZ PSC 833. The increase effect of SDZ PSC 833 is consistent with the hypothesis of a saturation of the P-gp pump present at the BBB. The involvement of P-gp in the brain passage of SDZ PSC 833 could be of great significance for clinical application of the drug in the treatment of brain cancers when it is given in combination with anticancer agents.

Because the significance of P-gp in the *in vivo* secretion of β -blockers in intestinal epithelial cells is unclear, the secretory mechanism for β -blockers and other drugs has been evaluated. Uptake of the β -blockers acebutolol, celiprolol, nadolol, and timolol, and the antiarrhythmic agent, quinidine by the multidrug-resistant leukemic cell line variant K562/ADM was significantly lower than that by drug-sensitive K562 cells, suggesting that these β -blockers are transported by P-gp out of cells. The reduced uptake of acebutolol by the drug-resistant K562/ADM cells was reversed by treating the cells with anti-P-gp monoclonal antibody, MRK16 whereas no such alteration in uptake was observed for drug-sensitive K562 cells. Acebutolol uptake by K562/ADM cells was, moreover, markedly enhanced, in a concentration-dependent manner; in the presence of the specific P-gp inhibitors, MS-209 and cyclosporin, Caco-2 cells were used for evaluation of the role of P-gp in intestinal permeability to drugs *in vitro*. Basolateral to apical transport of acebutolol was twice that in the reverse direction. A similar polarized flux was also observed in the transport of vinblastine, but not in that of acetamide or mannitol. When *in vivo* intestinal absorption was evaluated by the rat jejunal loop method, with simultaneous IV administration of a P-gp inhibitor, cyclosporin, intestinal absorption of both acebutolol

and vinblastine increased 2.6- and 2.2-fold, respectively, but no such enhancement was observed in the absorption of acetamide. The effect of cyclosporin on the intestinal absorption of several drugs was further examined, and the extent of the contribution of P-gp as an absorption barrier to those drugs was evaluated. ATP depletion by occlusion of the superior mesenteric artery resulted in a clear increase in epithelial permeability to vinblastine, but not to 3-*O*-methylglucose or acetamide, indicating that vinblastine is secreted by ATP-dependent P-gp into the lumen. These findings demonstrate that P-gp plays a role as an absorption barrier by transporting several drugs from intestinal cells into the lumen.

6. The role of intestinal cytochrome P450 (CYP450) and P-gp in metabolism and bioavailability of xenobiotics

CYP450 and P-gp are important detoxifying systems that protect us from many potential harmful xenobiotics. On the other hand, their activity also strongly limits the absorption of a wide variety of drugs. CYP450 enzymes and conjugation metabolic enzymes have been identified in human small intestine (Galetin et al., 2010). CYP isozymes in the small intestine account for approximately CYP3A, 80% (59%–96%), CYP2C9, 14%, CYP2C19, CYP2J2, and CYP2D6, <2% of total P450s (Paine et al., 2006). The total CYP450 in the intestine is ~1% of the estimated level (Paine et al., 1997). Several UDP-glucuronosyltransferase (UGT) and sulfotransferase conjugation enzymes are also expressed in human intestine (Tukey and Strassburg, 2001; Lindsay et al., 2008). Furthermore, the intestine also contains efflux transporters, in particular, P-gp that can decrease intestinal CYP3A first-pass metabolism by decreasing the intracellular concentration of drugs and metabolites by active efflux (Kivisto et al., 2004). In addition, MRP1 and MRP2 transporters are present in the intestine (Endres et al., 2006). CYP3A4 plays an important role in drug–drug interactions because of its high abundance in both liver and small intestines, and because it is susceptible to induction and inhibition by a number of chemicals (Ito et al., 2004). Furthermore, outflow of drugs from enterocytes is less than that from hepatocytes, because of the low blood flow in the intestinal mucosa; this leads to more efficient reactions by CYP3A4 than in the liver (Gomez et al., 1995). Drug–drug interactions are complex for mutual P-gp and CYP3A4 substrates (e.g., cyclosporine). Grapefruit selectively inhibited intestinal CYP3A4 metabolism, leading to removal of the role of the small intestine (Ducharme et al., 1995). In contrast, induction of intestinal CYP3A4 metabolism by rifampicin resulted in 8.5-fold greater AUC after administration of nifedipine (Holtbecker et al., 1996).

CYP3A4 and P-gp have an extensive overlap in their substrate spectrum. Thus, combined activity of CYP3A and P-gp results in a very efficient intestinal first-pass metabolism of orally administered drugs. Thus, for many shared P-gp and CYP3A substrate drugs, the combination of back-transport by P-gp in the intestinal epithelial cell and the presence of CYP3A4-mediated metabolism within the same cell results in a very efficient intestinal first-pass metabolism of orally administered drugs (Fan et al., 2010).

A recent study reported that Splenda (containing 1.1% sucralose) increased the expression of intestinal P-gp and two CYP450 isozymes, in male Sprague–Dawley rats (Abou-Donia et al., 2008) at levels that have been shown to decrease oral bioavailability of pharmaceutical drugs. Thus, ingestion of 300 mg/kg Splenda (3.1 mg/kg/day sucralose, a level currently approved for use in foods by the U.S. FD) for 90 days resulted in an increase of 2.43- and 1.4-fold in the expression of P-gp and CYP450, respectively. These results are similar to those found in a clinical study of an increase of 1.4-fold of P-gp following administration of St. John's wort (an herbal medicine commonly used as an antidepressant) for 14 days in healthy human subjects and subsequent 18% decrease in absorption of digoxin, a P-gp substrate that undergoes minimal metabolism (Dürr et al., 2000). Also, Splenda-induced increase in CYP450 expression was similar in magnitude to that induced by St. John's wort that is known to reduce oral bioavailability of many drugs including cyclosporin, indinavir, and amitriptyline (Dürr et al., 2000). Increased expression of P-gp results in decreased oral bioavailability of many anticancer drugs such as anthracyclines (doxorubicin and daunorubicin) and vicar alkaloids (vinblastine and vincristine) through the phenomenon of MDR that is associated with the chronic use of anticancer drugs, that is, increased efflux lowers intracellular drug concentration in neoplastic tissues (Gottesman et al., 2002). CYP450 enzymes are involved in the metabolism of a large number of drugs (Rendic and DiCarlo, 1997; Michalets, 1998; Clark and Jones, 2002); thus, Splenda-increased expression of CYP isozymes could accelerate drug metabolism leading to reduced clinical efficacy.

The action of the chlorocarbon sucralose, the active ingredient of Splenda on both P-gp and CYP isozymes, is consistent with previous reports that chlorinated hydrocarbons interact with both proteins resulting in their increased expression (Abou-Donia and Menzel, 1968; Bain and LeBlanc, 1996; Lanning et al., 1996; Bois et al., 1998; Poet et al., 2003; Leslie et al., 2005). The mechanism by which Splenda increases expression of P-gp and CYP450 isozymes may take place via the nuclear pregnane

X receptor (PXR). Several studies have shown that PXR regulates gene expression of CYP3A4 and the *MDR1* gene that encodes P-gp (Geick et al., 2001; Tompkins and Wallace, 2007). Also, the PXR receptor was shown to be activated by organochlorine compounds (Kliwer, 2003; Jacobs et al., 2005). This increased expression of P-gp and CYP isozymes may explain the finding that 65%–95% of orally administered sucralose was not absorbed from the gastrointestinal tract (Federal Register, 1998). These results suggest that orally administered sucralose is subjected to intestinal first-pass metabolism via two processes: (1) extrusion by P-gp from the intestinal walls back to the lumen and (2) metabolism via CYP isozymes in intestinal walls. The sum of these two processes explains why the lipid-soluble sucralose (Miller, 1991; Yotka et al., 1992; Wallis, 1993) is sequestered in the intestine and only a very small portion of it is absorbed into circulation.

In the small intestine, P-gp located in enterocytes might reduce the exposure of microsomal (intracellular) CYP3A to orally administered drugs. In contrast, in hepatocytes, orally administered drugs would first encounter (following uptake into the liver) intracellular CYP3A, before being potentially transported into the bile by P-gp. Numerous studies have shown that CYP3A enzymes are involved in the metabolism of ~50% of drugs (Guengerich, 1999). These enzymes are also involved in the synthesis of endogenous substrates, including cholesterol, steroid hormones, bile acids, and vitamin D (Xu et al., 2006). Oral drug administration has many advantages: patient-friendly, cost-effective, and safer than IV administration. Thus, it is advantageous to enhance oral bioavailability by inhibiting CYP3A and/or P-gp. For example, the anticancer drug docetaxel has very low oral bioavailability because it is a very good substrate for both CYP3A and P-gp. Recent study using simultaneous administration of docetaxel and ritonavir (an inhibitor for CYP3A and P-gp) resulted in enhanced oral bioavailability of docetaxel to the same range achieved by IV administration without ritonavir (Oostendorp et al., 2009). It will often be necessary to simultaneously inhibit both CYP3A and P-gp to obtain a substantial gain in oral bioavailability. This consideration will need to be taken into account to improve oral bioavailability of effective (anticancer) drugs that suffer from poor oral bioavailability because they are shared substrates of P-gp and CYP3A4. It is noteworthy that drugs that are not P-gp substrates such as triazolam, intestinal CYP3A-dependent metabolism can be more significant than hepatic metabolism after oral administration (van Waterschoot et al., 2011).

Recent studies with tissue-specific CYP3A4 transgenic mice have shown that intestinal CYP3A4 can regulate the expression of several detoxifying enzymes in the liver. Thus, long-term specific inhibition of intestinal CYP3A4 activity by grapefruit juice could result in higher levels of detoxifying enzymes (including CYP3A4) in the liver. It is noteworthy that although strategies to simultaneously inhibit CYP3A and P-gp can improve the oral drug bioavailability, they can give rise to toxicities that have not been previously encountered (Lemaire et al., 1966).

7. P-gp in nonmammalians

a. Bacteria

Using of a prokaryote such as *Escherichia coli* to study mammalian P-gps has two advantages. First, if overexpression can be achieved (El-Masry and Abou-Donia, 1999; 2000), the purification of large amounts of P-gp for biochemical reconstitution and structural studies is possible, and could be achieved more rapidly than if carried out in animal cells (Terao et al., 1996). Earlier studies suggested that the glycosylation is dispensable for P-gp function, so that unglycosylated P-gp expressed in bacteria should be functional. Second, if even low-level expression of functional P-gp can be achieved in intact cells, the short generation time, ease of drug selection, and ease of plasmid manipulation associated with *E. coli* offers tremendous advantages. An example of a successful high-level overexpression of higher eukaryotic membrane proteins in *E. coli* was the 55 kDa bovine 17 α -hydroxylase cytochrome P-450 from microsomes, containing a single predicted transmembrane helix, expressed up to 16 mg/L of *E. coli* culture.

b. Yeast

Heterologous expression of P-gps in the yeast *Saccharomyces cerevisiae*, a simple eukaryote, has many of the same advantages of *E. coli*. The potential for overexpression is perhaps greater in yeast than in *E. coli* due to the presence of eukaryotic membrane biogenesis and trafficking machinery much more like that of the homologous P-gp system. The biochemical and bioenergetic characteristics of P-gp expressed in yeast have been extensively explored by Gros et al. (1986) in the last 4 years. Recently, used the NDH promoter, expressed mouse P-gp (*mdr3*) in inside-out plasma membrane vesicles, and demonstrated ATP-dependent, osmotically sensitive transport of vinblastine and colchicine, which was inhibitable by verapamil. The transport properties of a variant of mouse P-gp (*mdr3*) bearing the (Ser939–Phe) mutation were quantitatively and qualitatively similar to those in animal cells. Unfortunately, the basal or drug-stimulated ATPase activities of P-gp could not be detected in this system.

TABLE 17.19
P-gp Genes

Species	Drug Transporters	Phospholipid Translocators
Human	MDR1	MDR2, MDR3
Mouse	mdr3/mdr1a mdr1/mdr1b	mdr2
Hamster	pgp1 pgp2	mdr3
Rat	mdr1a mdr1b	mdr2

TABLE 17.20
Representative of P-gp Substrates

Anticancer Agents	Other
Actinomycin D	Celiprolol
Colchicine	Cortisol
Daunorubicin	Digoxin
Docetaxel	Diltiazem
Doxorubicin	Erythromycin
Etoposide	Estrogen glucuronide
Mitomycin C	Gramicidin D
Mitoxantrone C	Indinavir
Paclitaxel	Ivermectin
Teniposide	Loperimide
Topotecan	Morphine
Vinblastine	Nifedipine
Vincristine	Neflinavir
VP-16	Progesterone
	Rifampicin
	Saquinavir
	Terfenadine

TABLE 17.21
Relative mRNA Expression of P-gp

Relative mRNA Expression	Tissue
High	Kidney, adrenal glands, liver, lung
Intermediate	Jejunum, colon, bone membrane, rectum
Low	Brain, prostate, muscle, skin, spleen, bone marrow.

TABLE 17.22
Partial List of MDR Reversal Agents

Amidodaron	Quinidine
Cremophor EL	Quercetin
Cyclosporin A	Rapamycin
FK506	Reserpine
Trans-flupenthixol	Staurosporine
Genistein	Tamoxifen
Ketoconazole	TPGS
Progesterone	Trifluoroperazine
Verapamil	

PLACENTA: METABOLISM AND TRANSPLACENTAL TRANSFER OF XENOBIOTICS

Placenta is a semipermeable barrier that separates the mother and the fetus. It is essential for the growth and development of the embryo and fetus during pregnancy. It regulates the exchange of nutrients, gases, waste, and endogenous and foreign molecules between maternal and fetal circulations to support fetal growth and development. During early gestation, the placenta mediates the implantation of the embryo in the uterus and produces hormones that prevent the end of ovarian cycle (Pasqualini and Kincl, 1985). After implantation, it regulates the supply of oxygen and nutrients to the fetus. It also produces various hormones to stimulate maternal blood cell production, increase blood volume, and regulate appetite. In addition, the placenta functions to remove waste and toxic products from the fetus and metabolism.

Fetal exposure to thalidomide during 24–36 days of pregnancy from 1957 to 1961 that resulted in teratogenicity in the offspring with deformed limbs proved that the placenta is highly permeable to xenobiotics. Fetuses are chronically exposed to food and chemicals. Placental epithelia expressing xenobiotic metabolism enzymes and protein transporters are involved in the regulation of chemicals in fetal environment.

The human placenta contains several enzymes responsible for both Phase I and Phase II metabolism that seems minor compared to the liver (Prouillac and Lecoecur, 2010). The following CYP450 have been isolated from the placenta during the first trimester of pregnancy: CYP1A1, CYP1A2, CYP2D6, CYP3A4, CYP3A5, and CYP3A7. The following enzymes were isolated from human placenta from a full-term placenta: CYP1A1, CYP1B1, CYP2E1, CYP2C9, CYP2F1, CYP3A4, CYP3A5, CYP3A7, CYP4B1, CYP11A1, and CYP19. The quantities of these enzymes vary with placental development, length of gestation, and maternal health. The activities of these enzymes decrease from the first trimester of pregnancy to the second and third. In placenta, the following enzymes are involved in steroid hormones synthesis and metabolism: 3-hydroxysteroid dehydrogenase, aromatase, and 17 β -hydroxysteroid dehydrogenase (type 1 and type 2). Phase II enzymes in the placenta include glutathione *S*-transferase α and π , epoxide hydrolase, *N*-acetyl transferase, sulfotransferases isoforms, and hydroxysteroid deshydrogenase.

Most xenobiotics cross the human placental via passive diffusion. Facilitated diffusion, phagocytosis, and pinocytosis are less important for placental drug transfer (Syme et al., 2004). P-gp plays an important role in the mouse placenta for carrier-mediated transport of xenobiotics (Lankas et al., 1998). The human term placenta expresses at least three members of MDR resistance-associated proteins (MRP) family: MRP1, MRP2, and MRP3. The expression of breast cancer–resistance protein (BCRP) is

higher in human placenta than in other organs (Allikmets et al., 1998). BCRP structure suggests that, like P-gp, it may have a protective role in removing cytotoxic drugs from fetal tissues.

TOXICOKINETICS

Toxicokinetics is defined as the study of toxicant movement. It is concerned with the rates of all metabolic processes including absorption, distribution, biotransformation, binding, and elimination. Toxicokinetic studies are carried out by measuring the concentration of xenobiotics in various tissues and body fluids over time.

Toxicokinetic studies consider the body as a system of compartments. A compartment is defined by the organs, tissues, cells, and fluids that share similar rates of uptake and clearance of a xenobiotic. Chemicals equilibrate rapidly in the central compartment which may include blood and tissue with profuse blood supply, example, liver, kidney, lung, and heart. On the other hand, slow equilibrating compartments, known as peripheral compartments, may include tissues with poor blood supply, example, muscle, adipose tissues, and bones. It is important to know various blood components: blood is the fluid portion of blood plus formed elements (white cells, red cells, and platelets); plasma is the fluid portion of blood (including soluble proteins, but not formed elements); and serum is the remaining product when the soluble protein fibrinogen is removed from plasma.

KINETIC ORDER OF ELIMINATION REACTION

The elimination of xenobiotics may follow a zero- or a first-order reaction.

1. Zero-order kinetics

Zero-order reactions are characterized by

- The processes of elimination are saturated.
- Rate of elimination remains constant and is independent of concentration or amount of xenobiotics.
- The drug is being cleared as fast as possible.
- The biological system is rate limiting.
- The half-life ($t_{1/2}$) increases with dose.
- A plot of drug concentration vs. time is linear.
- Example: Ethanol

The body can metabolize 10 mL/h of ethanol which is one beer or one mixed drink per hour. Thus, if one drinks a six pack of beer (60 mL ethanol) in 1 h, the following kinetics would occur (Table 17.23).

2. First-order kinetics

First-order reactions are characterized by

- Rate of elimination of a chemical (dX/dt) from the body is directly proportional to the concentration

(c) or amount (X) of chemical present at that time (t) as shown in Equation 17.1:

$$\frac{dX}{dt} \propto X \quad \text{or} \quad \frac{dX}{dt} = -aX \quad (17.1)$$

where “ a ” is a constant, the negative sign indicates that the xenobiotic is being lost from the body. Integration of Equation 17.1 and evaluation of the constant of integration at $t = 0$ where $X = X_0$ (the administered dose) gives Equation 17.2:

$$X = X_0 e^{-at} \quad (17.2)$$

Equation 17.2 describes the content of the chemical (X) at time (t). Taking the natural logarithm of Equation 17.2 gives a straight line equation (17.3):

$$\ln X = \ln X_0 - at \quad (17.3)$$

or

$$\log X = \log X_0 - \frac{kt}{2.303} \quad (17.4)$$

A plot of $\log X$ against time (t) gives a straight line with slope ($-a$) and ordinate intercept of $\log X_0$. The slope (a) can be determined from the line that equals $k/2.303$, where k is the apparent first-order elimination rate constant for body content of the xenobiotic. It is easier, however, to determine (a) from the relationship:

$$k = \frac{0.693}{t_{1/2}} \quad (17.5)$$

Then,

$$\text{Slope} = \frac{k}{2.303} = \frac{\log C_2 - \log C_1}{t_2 - t_1} \quad (17.6)$$

where

C_1 and C_2 are plasma concentrations at times t_1 and t_2 , respectively

$t_{1/2}$ is the biological or elimination half-life of xenobiotic in the body

k is the fraction of dose eliminated per unit time

- In the first-order process, the chemical is rate limiting.
- The $t_{1/2}$ is independent of dose.
- Most xenobiotics are handled by the body by first-order processes.

- e. At high chemical concentrations, the biological systems may become saturated and change from first-order to zero-order kinetics.
- f. The elimination rate constant k is defined as the amount or concentration changes by some fraction per unit time. Thus, a k of 0.2/h for a xenobiotic means that 20% of the chemical is eliminated per hour. An example of a xenobiotic behavior according to first-order reaction is shown in Table 17.24.

3. Determination of the kinetic order

To determine if the elimination process follows a zero- or first-order kinetics, the fraction of the chemical left in body (fraction of dose) or plasma concentration is graphed at varying times of administration on rectilinear or semilog paper. Zero-order kinetics results in a straight line on rectilinear paper and first-order kinetics is linear in semilog (Figure 17.3).

TABLE 17.23
Zero-Order Kinetic for Elimination of Ethanol

	Hours after Ethanol Consumption					
	1	2	3	4	5	6
Ethanol eliminated (mL)	10	10	10	10	10	10
Ethanol remaining (mL)	50	40	30	20	10	0
Ethanol eliminated (%)	17	20	25	33	50	100
(% of total ethanol)	(10/60)	(10/50)	(10/40)	(10/30)	(10/20)	(10/10)

TABLE 17.24
First-Order Process of a Xenobiotic

	Time after Uptake, h								
Xenobiotic	0	1	2	3	4	5	6	7	8
Chemical eliminated (mg)	0	20	16	12.8	10	8.2	6.6	5.2	4.2
Chemical remaining (mg)	100	80	64	51	41	33.0	26	21	16.8
Chemical eliminated		20/100	16/80	12.8/63.8	10/51	8.1/41.2	6.6/32.6	5.2/26.2	4.3/21.0
(% of remaining)		20	20	20	20	20	20	20	20

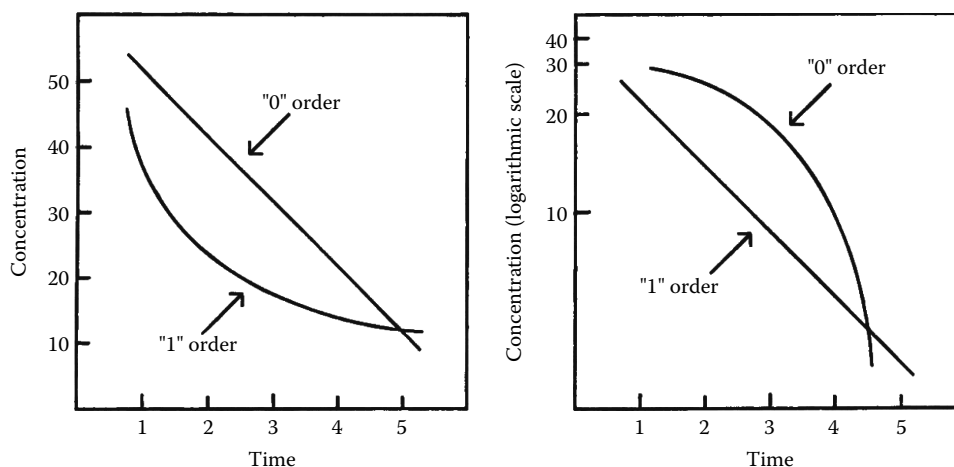


FIGURE 17.3 Determination of the kinetic order: “0” and “1” order.

BODY COMPARTMENTS

Following its absorption, a xenobiotic is in the blood and is simultaneously distributed through the body, undergoes metabolic biotransformation, and is eliminated. Distribution process is much more rapid than elimination, and is carried out by the circulation and is affected by regional blood flow. If a xenobiotic is present in the body in several tissues or organs, and if it passes from one location to another at different rates, then each location is considered a separate compartment for the xenobiotic.

1. One-compartment open-model system

In this model, the body is considered as a single compartment in which the xenobiotic equilibrates instantaneously. In this instance, all body tissues and fluids are in rapid equilibrations within the blood, and the concentration of the xenobiotic is constant throughout.

Whereas the log fraction of xenobiotic in the body or its log concentration in the blood is plotted against time, a straight line is obtained indicating that its elimination process follows first-order kinetics (Figure 17.4). The reason why xenobiotics are mono-exponential or monophasic is that they equilibrate into tissues very fast without significant storage or binding. In this model, the xenobiotic is eliminated from the body by apparent first-order kinetics where the rate of elimination is proportional to its concentration in plasma at that time as described in Equation 17.7:

$$\frac{dC}{dt} = -kC \quad (17.7)$$

where

C is the concentration of the chemical in the plasma at time t

k is the apparent first-order elimination rate constant for the xenobiotic, and the negative sign indicates that the chemical is being lost from the plasma

Integration of Equation 17.7 describes the course of the xenobiotic concentration in plasma as shown in Equation 17.8:

$$C = C_o e^{-kt} \quad (17.8)$$

or

$$\ln C = kt + \ln C_o \quad (17.9)$$

or

$$\log C = \frac{-kt}{2.303} + \log C_o \quad (17.10)$$

where C_o is the xenobiotic concentration in the plasma at time zero. A plot of $\log C$ vs. time yields a straight line with slope $= -k$ and intercept $= C_o$.

a. Toxicokinetic parameters

i. Elimination half-life

The elimination half-life, $t_{1/2}$, is the time required to decrease plasma concentration of a chemical to half of its original value, assuming that the chemical is eliminated by a first-order process. Its unit is time:

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} = \frac{0.693 \times V_d}{Cl} \quad (17.11)$$

where

V_d is the apparent volume of distribution

Cl is total clearance

Elimination half-life ($t_{1/2}$) is useful in determining the length of the period before multiple dosing would reach steady state. Table 17.25 demonstrates that it takes 4–5 half-life values to reach the steady state.

ii. Apparent volume of distribution (V_d)

The apparent volume of distribution (V_d) represents the relationship of concentration of chemical in plasma and amount in body. It is defined as the apparent volume to which the xenobiotic is distributed in the body:

$$V_d = \frac{\text{Amount of chemical in body}}{\text{Plasma concentration}} \quad (17.12)$$

To obtain V_d , plasma concentration at time zero intercept (C_o) is divided into applied dose (D_o) as described in Equation 17.13:

$$V_d = \frac{\text{Dose}}{C_o} \quad (17.13)$$

V_d has a volume unit of mL or L or adjusted for body weight, example, L/kg.

The value of V_d gives some information about the xenobiotic in the body rather than representing a real body space. V_d increases as the distribution into the body increases.

Body fluids in a 70 kg man (100%):

- Fluid (water): 60% (42 L) composed of
 - Intracellular fluid: 40% (28 L)
 - Extracellular fluid: 20% (14 L)
 - Plasma: 4% (3 L)
 - Interstitial fluid: 16% (11 L): Interstitial fluid surrounds cells outside the vascular system

Volume of distribution of a drug in a 70 kg man:

- (1) 15–18 L: Distribution in extracellular fluid
- (2) 40 L: Distribution into all body water
- (3) 40–50 L: Distribution is concentrated in tissues outside the plasma and interstitial fluid
- (4) A drug that accumulates in tissues, example, fat tissues, will have low plasma concentration and high V_d
- (5) Drugs with very small V_d (<10 L) are confined to extracellular fluids (plasma and interstitial fluid) because
 - The molecule is too large to leave this compartment.
 - The molecule binds preferably to plasma proteins (e.g., to albumin).
 - A drug cannot enter cells because of its low lipid solubility; it distributes in the extracellular compartment and has small V_d (12–20 L).
- (6) Bone is a reservoir for drugs such as tetracycline and heavy metals that have large V_d

Clinical implications of apparent volume of distribution (V_d):

The value of V_d does not represent a real body space, but rather provides information about the distribution of the chemical in the body. As the distribution into tissues increases, V_d increases and plasma concentration decreases. If the chemical is widely distributed or has a high affinity for a tissue such as fat, V_d may be >1000 mL/kg. V_d also reflects the fraction of the chemical that is available for excretion and action. Thus, if a chemical is extensively distributed in the tissues, as indicated by large V_d , less will be available in plasma for excretion. If V_d is small, indicating less tissue distribution, more of it will be available in plasma for excretion. A drug with large V_d is extensively distributed in tissues, less of the drug in plasma, and less available to exert its action. The volume of distribution is important in determining the proper drug dosing. Generally, dosing is proportional to the volume of distribution; thus, to achieve the desired dose, a larger dose must be used for a drug with large volume of distribution. Factors affecting

V_d are lipid vs. water solubility of drug, plasma volume, binding to tissues, plasma protein volume, and obesity (total body weight).

If a xenobiotic is not well distributed in the tissues, then its distribution is limited to plasma, extracellular fluid, or total body water and resulting in V_d values of 40, 170, and 580 mL/kg, respectively. On the other hand, if the chemical is thoroughly distributed or has a high affinity for a tissue such as fat or muscle, V_d may be >100 mL/kg. V_d also produces an indication of the fraction of the chemical that is available for elimination. A large V_d is an indication that the chemical is extensively distributed in the tissues, and therefore less of it will be available for excretion. A small V_d indicates less tissue distribution, and more of the chemical will be available for excretion. V_d may be calculated using Equation 17.14:

$$V_d = \frac{D_{IV}}{AUC \times k} \quad (17.14)$$

where

D is the dose following an IV injection

AUC is the total area under plasma concentration vs. time course after a single dose

k is the elimination rate constant which can be obtained from the following equation: slope = $-k/2.303$

AUC can be obtained by

- (a) Planimeter (rectilinear paper)
- (b) Cut and weight (rectilinear paper)
- (c) Trapezoidal rule

$$A = \frac{1}{2} (C_1 + C_2) \times (T_2 - T_1) \dots + \frac{C^*}{k} \quad (17.15)$$

where

C^* is the last plasma concentration time point

C_1 and C_2 are the plasma concentrations at t_1 and t_2 , respectively

Volume of distribution of some common drugs is listed in Table 17.26.

iii. Total clearance

Clearance (Cl) is defined as the volume of the central compartment which is cleared of drug in unit time. It is a measure of the removal of the drug from the body. Therefore, clearance (expressed as volume/time) measures the efficiency with which a chemical is eliminated from the body by all routes. Clearance does not indicate the amount of drug being removed; it indicates the volume of plasma (or blood) from which the drug is being completely removed, or cleared in a given time period. Clearance may be determined by Equation 17.16:

$$Cl = V_d \times k = \frac{0.693}{t_{1/2}} V_d \quad (17.16)$$

Clearance is usually calculated according to Equation 17.17:

$$Cl = \frac{\text{Dose}}{\text{AUC}} \quad (17.17)$$

Total clearance includes clearance of all organs, example, liver (hepatic) clearance, (Cl_H), kidney (renal) clearance (Cl_R), biliary clearance (Cl_b), and other routes (Cl_{other}) such as gastrointestinal tract, pulmonary, etc. Total body clearance (Cl_T) of a drug is the sum of all the clearance routes:

$$Cl_T = (Cl_H + Cl_R + Cl_b + Cl_{\text{other}})$$

Clearance by any organ is determined by the blood flow through the organ, Q , and the extraction ratio, E , according to Equation 17.18:

$$Cl = Q \times E \quad (17.18)$$

E is determined by the inflowing, C_{in} , and outflowing, C_{out} , concentration of the xenobiotic (Equation 17.19):

$$E = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \quad (17.19)$$

This ratio must be a fraction between zero and one. Organs that are very efficient in eliminating a drug will have an extraction

ratio approaching one (100% extraction). Rating of extraction ratios (E) are listed as follows:

Extraction ratio (E) rating

> 0.7 High

0.3–0.7 Intermediate

< 0.3 Low

The drug clearance of any organ is determined by blood flow and the extraction ratio:

Organ clearance

= blood flow \times extraction ratio

or

$$Cl_{\text{organ}} = Q \times C_{\text{in}} - \left(\frac{C_{\text{out}}}{C_{\text{in}}} \right) \text{ or } Cl_{\text{organ}} = QE.$$

Renal clearance (Cl) may also be determined according to the following equation:

$$Cl = \frac{\text{Urine flow} \times \text{concentration in urine}}{\text{Plasma concentration}} = \text{mL/min} \quad (17.20)$$

The maximum value for an organ clearance is that of its blood flow rate. Thus, hepatic and renal clearance cannot exceed their blood flow rates of 1500 and 650 mL/min, respectively, in humans. High hepatic clearance results in “first-pass” effect, that is, high biotransformation elimination of the chemical after oral administration, example, chlorpyrifos.

Clearance unit is volume per time, example, mL/min or L/h, or expressed for body weight, example, L/h/kg. To determine clearance, urine should be collected over at least five elimination half-lives to maximize recovery of dose. Another example is propranolol that is eliminated exclusively by hepatic metabolism. The extraction ratio for propranolol is greater than 0.9, indicating that most of the drug is removed by one pass through the liver.

Therefore, clearance is approximately equal to liver blood flow. This explains that the oral dose of this drug is 10–20 times the equivalent IV dose.

Total (Cl_T), hepatic (Cl_H), and renal (Cl_R) clearance values are good indicators for the elimination processes of the xenobiotic, and consequently its toxicity. High total and hepatic clearance values are consistent with high extraction values by the liver. This suggests that patients with liver diseases would have less total and hepatic clearances resulting in high systematic availability and more toxicity. High renal clearance, example, 100 mL/min suggests that the xenobiotic will accumulate in patients with renal failure because less of the chemical will be eliminated via the kidney.

iv. Bioavailability

Bioavailability, F , is the fraction of the dose that is absorbed into the systemic circulation. It is calculated as the ratio of AUCs obtained following dosing other than IV and IV dosing according to Equation 17.21:

$$F = \frac{AUC_n \times \text{Dose}_{IV}}{AUC_{IV} \times \text{Dose}_n} \quad (17.21)$$

n indicates dosing by a route other than IV dosing.

v. Relative residence

Relative residence (RR) of a xenobiotic in specific tissues is calculated according to Equation 17.22:

$$R_R = \frac{AUC_{\text{organ}}}{AUC_{\text{plasma}}} \quad (17.22)$$

Thus, the R_R values reflect the relative accumulation of a xenobiotic in specific organ or the relative exposure of individual organs to the xenobiotic. The use of this parameter avoids the time-dependent changes in concentration ratios. R_R is a time-independent parameter and, assuming linear kinetics, it predicts the ratio of average concentrations of the xenobiotic in specific organs, \bar{C}_{plasma} to those in plasma \bar{C}_{plasma} , or

$$R_R = \frac{\bar{C}_{\text{organ}}}{\bar{C}_{\text{plasma}}} \quad (17.23)$$

2. Two-compartment open-model system

After the introduction of xenobiotics into the central compartment, they undergo distribution in its highly perfused tissues. The concentration of some xenobiotics in these tissues, however, declines more rapidly during the distribution phase than the postdistribution phase. The chemical eventually distributes to less perfused tissues, example, muscle, skin, and fat, known as peripheral compartments. The chemical concentrations in the peripheral compartments will reach a maximum and then begin to decline during the elimination phase. As a result, the plasma concentration of the xenobiotic rapidly declines biphasically and, in some cases, polyphasically. The early phase is associated with the distribution of the chemical into tissues, and the last phase is associated with the elimination of the chemical after the distribution phases have been completed. An equilibrium is attained, with time, between the concentration of the chemical in the central and peripheral compartments. Chemicals pass into and put off each compartment by a first-order process, and are eliminated only from the central compartment by a first-order process.

In the two-compartment open-model system (Figure 17.5), k_{12} is the rate constant for the movement of the xenobiotic from the central compartment (1) to the peripheral compartment (2), k_{21} is the rate constant for the passage of the xenobiotic from the peripheral compartment back to the central compartment, and k_{10} is the rate constant of elimination from the central compartment.

The time course of the xenobiotic concentration in the two-compartment open-model system is described by the following equation:

$$C_t = A_0 e^{-\alpha t} + B e^{-\beta t} \quad (17.24)$$

C_t is plasma concentration of the chemical at time t .

The rate constant for the first phase of the biphasic decline of the plasma chemical concentration is α (Figure 17.5). It is calculated by the method of residuals as follows: the straight line of the β -phase is extrapolated back to time zero. The extrapolated values are subtracted from the experimental values of the xenobiotic concentration and the resultant values are plotted on semi-logarithmic paper.

These rate constants are calculated as follows:

$$k_{21} = \frac{A\beta + B\alpha}{A + B} \quad (17.25)$$

$$k_{10} = \frac{\alpha B}{k_{21}} \quad (17.26)$$

$$k_{12} = (\alpha + \beta) - (k_{21} + k_{10}) \quad (17.27)$$

Then α is estimated from the slope of the secondary plot according to the following equation:

$$\alpha = 2.303 \times \text{slope} \quad (17.28)$$

or from the half-time according to this relationship

$$\alpha = \frac{0.693}{t_{1/2}} \quad (17.29)$$

The rate constant for the second phase of the biexponential decline in the xenobiotic concentration is β .

A_0 is the intercept of the straight line obtained after extrapolating the secondary plot back to time zero.

B_0 is the intercept obtained by extrapolating the straight line associated with β back to time zero.

α -Phase is the rapid phase of the biphasic decline of the xenobiotic concentration. It is described by the following equation:

$$A = A_0 e^{-\alpha t} \quad (17.30)$$

β -Phase is the slow phase of the biphasic decline of the chemical concentration. It is described by the following equation:

$$B = B_0 e^{-\beta t} \quad (17.31)$$

$$\beta = \frac{0.693}{t_{1/2}} \quad (17.32)$$

$$\text{Cl, total body clearance} = \beta \times V_d$$

$$\text{Cl} = \frac{DF}{\text{AUC}_{\text{plasma}}} \quad (17.33)$$

$$V_d = \frac{DF}{\beta(\text{AUC})_{\text{plasma}}} = \frac{\text{Cl}}{\beta} \quad (17.34)$$

where F is bioavailability.

3. Three-compartment open-model system

In this system, it is assumed that all processes are linear and that elimination occurs from the central compartment. The xenobiotic is introduced into the central compartment which is connected to two peripheral “shallow” and “deep” compartments. The central compartment is formed of the plasma and highly perfused nonfat tissues such as blood cell, heart, lung, liver, kidney, and glands. The “shallow” peripheral compartment consists of poorly perfused tissues such as muscle, skin, and may include fatty tissues such as adipose tissues and bone marrow. The “deep” peripheral compartment contains tissues with negligible perfusion such as bone, teeth, cartilage, and hair.

Figure 17.6 shows schematic representations of the body as a three-compartment open model with xenobiotic eliminations from the central component. The constants k_{12} and k_{21} , and k_{31} and k_{13} are the apparent first-order rate constants for inter-compartmental transfer of the chemical between the shallow and central compartments, and deep and central compartments, respectively. The elimination rate constant from the central compartment is k_{10} .

Figure 17.6 represents a three-compartment system for plasma concentration curve following rapid IV administration of a xenobiotic.

The xenobiotic introduced into the central compartment is first rapidly distributed and equilibrated to well-perfused tissues, then slowly to tissues constituting the “shallow” compartment, and finally more slowly to the “deep” compartment. All processes are assessed to follow first-order kinetics. The plasma concentration curve appears triphasic and is described by the following equation:

$$C_p = P e^{-\Pi t} + A e^{-\alpha t} + B e^{-\beta t} \quad (17.35)$$

where

C_p is the xenobiotic concentration in the central compartment at the t

P , Π , A , α , B , and β are constants

The values of the three hybrid rate constants, Π , α , and β , reflect a rapid initial distribution of the chemical and a slower apparent elimination rate of the chemical from the body.

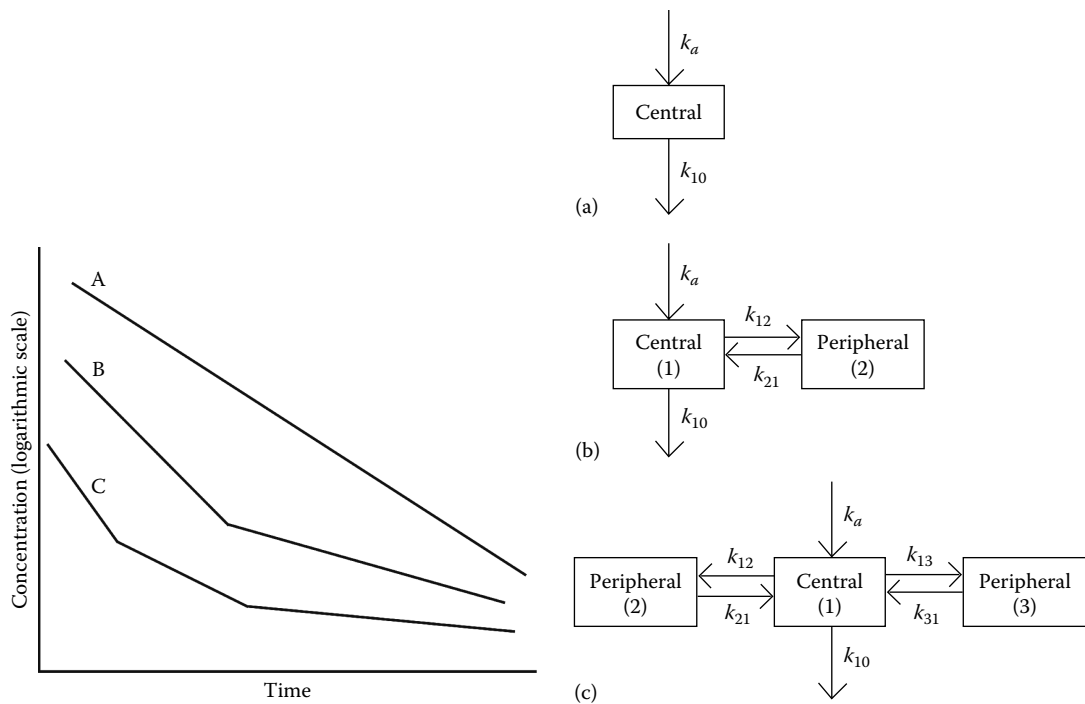


FIGURE 17.4 Xenobiotic plasma concentration (logarithmic scale) vs. time. (a) monoexponential, one-compartment; (b) biexponential, two-compartment; and (c) triexponential, three-compartment open-model system.

TABLE 17.25
Xenobiotic Steady State and Half-Life

Number of Half-Life	Xenobiotic Steady State (%)	Xenobiotic Left in Body (%)
1	50.00	50.00
2	75.00	25.00
3	87.50	12.50
4	93.75	6.25
5	96.87	3.13

TABLE 17.26
Apparent Volume of Distribution (V_d) of Some Common Drugs

Drug	% Plasma Protein Binding	Lipid Solubility/Tissue Binding	Volume of Distribution (L)
Warfarin	99	Low	10
Salicylic acid	90	Low	12
Gentamicin	<10	Low	18
Ethanol	—	Distribution in total body water	30
Theophylline	40	Low/medium	34
Diazepam	99	High	77
Digoxin	25	High	490
Chloroquine	61	High	8050
Amitriptyline	95	High	1050

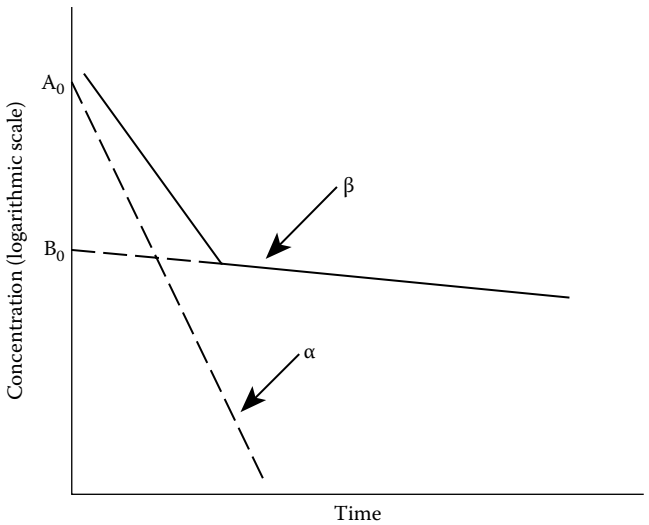


FIGURE 17.5 Biexponential decline of a xenobiotic concentration in plasma with time.

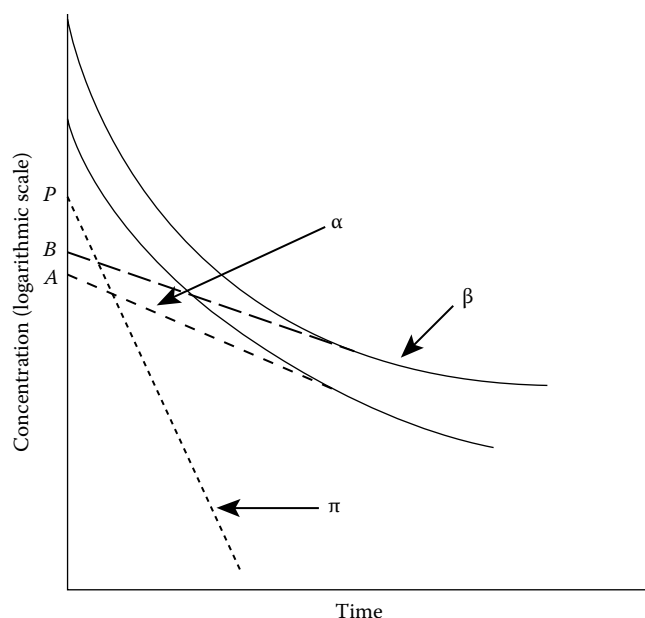


FIGURE 17.6 Schematic presentation of the three-compartment open model system.

REFERENCES

- Abou-Donia, M.B., Pharmacokinetics of a neurotoxic oral dose of leptophos in hens. *Arch. Toxicol.*, 36, 103, 1976.
- Abou-Donia, M.B., Metabolism and pharmacokinetics of a single oral dose of *O*-4-bromo-2,5-dichlorophenyl-*O*-methylphenylphosphonothioate (leptophos) in hens. *Toxicol. Appl. Pharmacol.*, 55, 131, 1980.
- Abou-Donia, M.B., Disposition, metabolism, and toxicokinetics, in: *Neurotoxicology*, Abou-Donia, M.B., Ed., CRC Press, Boca Raton, FL, 1992, 256.
- Abou-Donia, M.B., El-Masry, H.H., Abdel-Rahman, A.A., McLendon, R.E., and Schiffman, S.S., Splends alters gut microflora and increases intestinal P-glycoprotein and cytochrome P-450 in male rats. *J. Toxicol. Environ. Health Pt. A*, 71, 1415–1429, 2008.
- Abou-Donia, M.B. and Menzel, D.B., The in vivo metabolism of DDT, DDD, and DDE in the chick by embryonic injection and dietary ingestion. *Biochem. Pharmacol.* 17, 2143–2161, 1968.
- Abou-Donia, M.B. and Nomeir, A.A., The role of pharmacokinetics and metabolism in species sensitivity of neurotoxic agents. *Fundam. Appl. Toxicol.*, 6, 190, 1986.
- Abou-Donia, M.B., Nomeir, A.A., Bower, J.H., and Makkaway, H.A., Absorption, distribution, excretion, and metabolism of a single oral dose of [¹⁴C]tri-*o*-cresyl phosphate (TOCP) in the male rat. *Toxicology*, 65, 61, 1990.
- Abu-Qare, A.W., Abdel-Rahman, A.A., Kishik, A., and Abou-Donia, M.B., Placental transfer and pharmacokinetics of a single dermal dose of [¹⁴C]methyl parathion in rats. *Toxicol. Sci.*, 53, 5, 2000a.
- Abu-Qare, A.W. and Abou-Donia, M.B., Urinary excretion of metabolites following a single dermal dose of [¹⁴C]methyl parathion in rats. *Toxicology*, 150, 119, 2000a.
- Abu-Qare, A.W. and Abou-Donia, M.B., Simultaneous determination of pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide), permethrin and metabolites in rat plasma and urine using high-performance liquid chromatography. *J. Chromatogr. B*, 749, 171, 2000b.
- Abu-Qare, A.W., Brownie, C., and Abou-Donia, M.B., Placental transfer and pharmacokinetics of a single oral dose of [¹⁴C] *p*-nitrophenol in rats. *Arch. Toxicol.*, 74, 188, 2000b.
- Aldridge, W.N., Serum esterases. I. Two types of esterases (A and B) hydrolysing *p*-nitrophenyl acetate, propionate and butyrate, and a method for their determination. *Biochem. J.*, 53, 110, 1953.
- Allikmets, R., Schriml, L.M., Hutchinson, A., RomanoSpica, V., and Dean, M., A human placenta-specific ATP-binding cassette gene (*ABCP*) on chromosome 4q22 that is involved in multi drug resistance. *Cancer Res.*, 58, 5337–5339, 1998.
- Ariyoshi, N., Oguri, K., Yoshimura, H., and Funae, Y., Metabolism of the highly persistent PCG congener, 2,4,5,2',4',5'-hexachlorobiphenyl, by human CYP2B6. *Biochem. Biophys. Res. Comm.*, 212, 455–460, 1995.
- Augustinsson, K.B., Multiple forms of esterases in vertebrate blood plasma. *Ann. N.Y. Acad. Sci.*, 94, 844, 1961.
- Bain, L.J. and LeBlanc, G.A., Interaction of structurally diverse pesticides with the human *MDR1* gene product P-glycoprotein. *Toxicol. Appl. Pharmacol.*, 141, 288–298, 1996.
- Baker, K.J. and Bradley, S.E., Binding of sulfobromophthalein (BSP) sodium by plasma albumin. Its role in hepatic BSP extraction. *J. Clin. Invest.*, 45, 281, 1966.
- Bakry, N. M., Salama, A.K., Aly, H.A., and Abou-Donia, M.B., Milk transfer, distribution, and metabolism of a single oral dose of [¹⁴CH₃S] methamidophos in Sprague-Dawley rats. *Toxicologist*, 10, 346, 1990.
- Baldwin, S.J., Bloomer, J.C., Smith, G.J., Ayrton, A.D., Clarke, S.E., and Chenery, R.J., Ketaconazole and sulphophenazole as the respective selective inhibitors of P4503a and 2C9. *Xenobiotica*, 25, 261–270, 1995.
- Bergmann, E., Segal, R., and Rimon, S. A new type of esterase in hog-kidney extract. *Biochem. J.*, 67, 481, 1957.
- Bloomer, J.C., Clarke, S.E., and Chenery, R.J., Determination of P4501A2 activity in human liver microsomes using [3-C-14-methyl] caffeine. *Xenobiotica*, 25, 917–927, 1995.
- Bois, F., Beney, C., Boumendjel, A., Mariotte, A.M., Conseil, G., and Di Pietro, A., Halogenated chalcones with high-affinity binding to P-glycoprotein: Potential modulators of multidrug resistance. *J. Med. Chem.*, 41, 4161–4164, 1998.
- Brodie, B.B. and Axelrod, J., The estimation of acetanilide and its metabolic products, aniline, *N*-acetyl-*p*-aminophenol and *p*-aminophenol (free and total conjugates) in biological fluids and tissues. *J. Pharmacol. Exp. Ther.*, 94, 22, 1948.
- Carrington, C.D. and Abou-Donia, M.B., Variation between three strains of rats: Inhibition of neurotoxic esterase and acetylcholinesterase by tri-*o*-cresyl phosphate. *J. Toxicol. Environ. Health*, 25, 259, 1988.
- Chabra, R.S., Pohl, R.J., and Fouts, J.R., A comparative study of xenobiotic metabolizing enzymes in liver and intestine of various species. *Drug Metab. Dispos.*, 2, 443, 1974.
- Clarke, S.E. and Jones, B.C., Human cytochromes P450 and their role in metabolism-based drug–drug interactions, in: *Drug–Drug Interactions*, Rodrigues, A.D. Ed., Marcel Dekker, New York, 2002, pp. 55–88.
- Conney, A.H., Pharmacological implication of microsomal enzyme induction. *Pharmacol. Rev.*, 19, 317, 1967.
- Crespi, C.L., Penman, B.W., Steimel, D.T., Smith, T., Yang, C.S., and Sutter, T.R., Development of a human lymphoblastoid cell line constitutively expressing human CYP1B1 cDNA: Substrate specificity with model substitutes and promutagens. *Mutagenesis*, 12, 83, 1997.

- Dehal, S.S. and Kupfer, D., Metabolism of the proestrogenic pesticide methoxychlor by hepatic P450 monooxygenases in rats and humans. Dual pathways involving novel *ortho* ring-hydroxylation by CYP2B. *Drug Metab. Dispos.*, 22, 937, 1994.
- Delaporte, E., Cribb, A.E., and Renton, K.W., Interferon-mediated changes in the expression of CYP1A1 in human B lymphoblastoid (AHH-1 TK+/-) cells. *Can. J. Physiol. Pharmacol.*, 73, 1692, 1995.
- Ducharme, M.P., Warbasse, L.H., and Edwards, D.J., Disposition of intravenous and oral cyclosporine after administration with grapefruit juice. *Clin. Pharmacol. Ther.*, 57, 485–491, 1995.
- Dürri, D., Stieger, B., Kullak-Ublick, G.A., Rentsch, K.M., Steinert, H.C., Meier, P.J., and Fattinger, K., St. John's wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. *Clin. Pharmacol. Ther.*, 68, 598–604, 2000.
- Dutton, G.J., in: *Concepts in Biochemical Pharmacology*, 2nd edn., Brodie, B.B. and Gillette, J.R., Eds., Springer-Verlag, Berlin, Germany, 1971, p. 378.
- El-Masry, E. and Abou-Donia, M., Reversal of P-glycoprotein mediated multidrug resistance in *Escherichia coli* by chlorpromazine. *Bull. Fac. Sci., Zagazig Univ.*, 21(2), 18, 1999.
- El-Masry, E. and Abou-Donia, M., Reversal of P-glycoprotein expressed in *Escherichia coli* by ascorbic acid. *Life Sci.*, 73, 981–991, 2000.
- Endres, C.J., Hsiao, P., Chung, F.S., and Unadkat, J.D., The role of transporters in drug interactions. *J. Pharm. Sci.*, 27, 501–517, 2006.
- Fan, J., Maeng, H.J., and Pang, K.S., Interplay of transporters and enzymes in the Caco-2 cell monolayer. I. Effect of altered apical secretion. *Biopharm. Drug Dispos.*, 31, 215–227, 2010.
- Federal Register. Food additives permitted for direct addition to food for human consumption: Sucralose. Food and Drug Administration, HHS, Final Rule April 3, 1998 (Volume 63, Number 64), Rules and Regulations, pp. 16417–16433, Department Of Health And Human Services, Food and Drug Administration, 21 CFR Part 172, Docket No. 87F-0086. <http://www.cfsan.fda.gov/~lrd/ft980403.html>
- Feldman, R.J. and Maibach, H.I., Absorption of some organic compounds through the skin of man. *J. Invest. Dermatol.*, 54, 399, 1970.
- Fitzsimmons, M.E. and Collins, J.M., Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small-intestinal cytochrome P4503A4. Potential contribution to first pass metabolism. *Drug. Metab. Dispos.*, 25, 256, 1997.
- Fouts, J.R. and Brodie, B.B., The enzymatic reduction of chloramphenicol, *p*-nitrobenzoic acid, and other aromatic nitro compounds in mammals. *J. Pharmacol. Exp. Ther.*, 119, 197, 1957.
- Galetin, A., Gertz, M., and Houston, J.B., Contribution of intestinal cytochrome P450-mediated metabolism to drug–drug inhibition and induction interactions. *Drug Metab. Pharmacokinet.*, 25, 28–47, 2010.
- Geick, A., Eichelbaum, M., and Burk, O., Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J. Biol. Chem.*, 276, 14581–14588, 2001.
- Gibson, J.E. and Becker, B.A., Demonstration of enhanced lethality of drugs in hypoexcretory animals. *J. Pharm. Sci.*, 56, 1503, 1976.
- Gomez, D.Y., Wachter, V.J., Tomlanovich, S.J., Herbert, M.F., and Benet, L.Z., The effects of ketoconazole on the intestinal metabolism and bioavailability of cyclosporine. *Clin. Pharmacol. Ther.*, 58, 15–19, 1995.
- Gottesman, M.M., Fojo, T., and Bates, S.E., Multidrug resistance in cancer: Role of ATP-dependent transporters. *Nat. Rev. Cancer*, 2, 48–58, 2002.
- Gros, P., Neria, Y., Croop, J., and Housman, D., Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature (London)*, 322, 728, 1986.
- Guengerich, F.P., Human cytochrome P450 enzymes. *Life Sci.*, 50, 1471, 1992.
- Guengerich, F.P., Cytochrome P450 3A4: Regulation and role in drug metabolism. *Ann. Rev. Pharmacol. Toxicol.*, 39, 1–17, 1999.
- Holtbecker, N., Fromm, M.F., Kroemer, H.K., Ohnhaus, E.E., and Heidemann, H., The nifedipine–rifampin interaction. Evidence for induction of gut wall metabolism. *Drug Metab. Dispos.*, 24, 1121–1123, 1996.
- Ito, K., Brown, H.S., and Houston, J.B., Database analysis for the prediction of *in vivo* drug–drug interactions from *in vitro* data. *Br. J. Clin. Pharmacol.*, 57, 473–486, 2004.
- Jacobs, M.N., Nolan, G.T., and Hood, S.R., Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicol. Appl. Pharmacol.*, 209, 123–133, 2005.
- Johnson, M.K., Improved assay for neurotoxic esterase of screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.*, 37, 113, 1977.
- Kaminsky, L.S., Fasco, M.J., and Guengerich, F.P., Production and application of antibodies to rat liver cytochrome P450. *Methods Enzymol.*, 74, 262, 1981.
- Kivisto, K.T., Niemi, M., and Fromm, M.F., Functional interaction of intestinal CYP3A4 and P-glycoprotein. *Fundam. Clin. Pharmacol.*, 18, 621–626, 2004.
- Kliwer, S.A., The nuclear pregnane X receptor regulates xenobiotic detoxification. *J. Nutr.*, 133, 2444S–2447S, 2003.
- La Du, B.N., Mandel, G.H., and Way, E.L., *Fundamental of Drug Metabolism and Drug Disposition*, Williams & Wilkins, Baltimore, MD, 1972.
- Lankas, G.R., Wise, D., Cartwright, C., Pippert, T., and Umbenhauer, D.R., Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod. Toxicol.*, 12, 457–463, 1998.
- Lanning, C.L., Fine, R.L., Sachs, C.W., Rao, U.S., Corcoran, J.J., and Abou-Donia, M.B., Chlorpyrifos oxon interacts with the mammalian multidrug resistance protein, P-glycoprotein. *J. Toxicol. Environ. Health*, 47, 395, 1996.
- Lee, W.M., Drug-induced hepatotoxicity. *N. Engl. J. Med.*, 349, 474–485, 2003.
- Lemaire, M., Bruehlisauer, A., Guntz, P., and Sato, H., Dose-dependent brain penetration of SDZ PSC 833, a novel multidrug resistance-reversing cyclosporin, in rats. *Cancer Chemother. Pharmacol.*, 38(5), 481–486, 1996.
- Leslie, E.M., Deeley, R.G., and Cole, S.P., Multidrug resistance proteins: Role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.*, 204, 216–237, 2005.
- Lindsay, J., Wang, L.L., Li, Y., and Zhou, S.F., Structure function and polymorphism of human cystolic sulfotransferases. *Curr. Drug Metab.*, 9, 99–105, 2008.
- Maibach, H.I., Feldman, R.J., Milby, T.H., and Serat, W.F., Regional variation in percutaneous penetration in man. *Arch. Environ. Health*, 23, 208, 1971.
- Mason, H.S., Mechanism of oxygen metabolism. *Adv. Enzymol.*, 19, 79, 1957.
- Michalets, E.L., Update: Clinically significant cytochrome P-450 drug interactions. *Pharmacotherapy*, 18, 84–112, 1998.

- Miller, G.A., Sucralose, in: *Alternative Sweeteners*, 2nd edn., Nabors, L.O. and Gelardi, R.C., Eds., Marcel Dekker, New York, 1991, 173–195.
- Milne, M.D., Schribner, B.N., and Craford, M.A., Non-toxic diffusion and excretion of weak acids and bases. *Am. J. Med.*, 24, 709, 1958.
- Nakajima, M., Yamamoto, T., Nunoya, K.I., Yokoi, T., Nagashima, K., Inoue, K., Funae, Y., Shimada, N., Kamataki, T., and Kuroiwa, Y., Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. *J. Pharmacol. Exp. Ther.*, 277, 1010, 1996.
- Nakamura, A., Hirota, T., Morino, A., Shimada, T., and Uematsu, T., Oxidation of risogladine by the CYP2C subfamily in the rat, dog, monkey, and man. *Xenobiotica*, 27, 995, 1997.
- Nebert, D.W. and Gonzalez, F.J., P450 genes. Structure, evolution, and regulation. *Annu. Rev. Biochem.*, 56, 945, 1987.
- Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyerisen, R., Gonzalez, F.J. et al., The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.*, 12, 1, 1993.
- Nomeir, A.A. and Abou-Donia, M.B., Disposition of tri-*o*-cresyl phosphate (TOCP) and its metabolites in various tissues of the male cat following a single dermal application of [14C] TOCP. *Drug Metab. Dispos.*, 12, 705, 1984.
- Omura, T. and Sato, R., The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.*, 239, 2370, 1964.
- Oostendorp, R.L., Huitema, A., Rosing, H., Jansen, R.S., Ter Hein, R., Keessen M., Beijnen, J.H., and Schellens, J.H., Coadministration of ritonavir strongly enhances the apparent oral bioavailability of docetaxel in patients with solid tumors. *Clin. Cancer Res.*, 15, 4228–4233, 2009.
- Othman, M.A. and Abou-Donia, M.B., Pharmacokinetic profile of (\pm)-gossypol in male Sprague–Dawley rats following single intravenous and oral administration. *Proc. Soc. Exp. Biol. Med.*, 188, 17, 1988.
- Paine, M.F., Hart, H.L., Lundington, S.S., Haining, R.L., Rettie, A.E., and Zeldin, D.C., The human intestinal cytochrome P450 “pie.” *Drug Metab. Dispos.*, 34, 880–886, 2006.
- Paine, M.F., Khalighi, M., Fisher, J.M., Shen, D.D., Kunze, K.L., Marsh, C.L., Perkins, J.D., and Thummel, K.E., Characterization of interintestinal and intrainestinal variations in human CYP3A-dependent metabolism. *J. Pharmacol. Exp. Ther.*, 283, 1552–1562, 1997.
- Pasqualini, J.R. and Kincl, F.A., Eds., Biosynthesis of metabolism of different hormones in the fetal placental compartments, *Hormones and Fetus*, Vol. I, Pergamon Press, Oxford, U.K., 1985, pp. 73–173.
- Poet, T.S., Wu, H., Kousba, A.A., and Timchalk, C., *In vitro* rat hepatic and intestinal metabolism of the organophosphate pesticides chlorpyrifos and diazinon. *Toxicol. Sci.*, 72, 193–200, 2003.
- Prouillac, C. and Lecoeur, S., The role of the placenta in fetal exposure to xenobiotics: Importance of membrane transporters and human models for transfer studies. *Drug Metab. Dispos.*, 38, 1623–1635, 2010.
- Rendic, S. and DiCarlo, F.J., Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab. Rev.*, 29, 413–580, 1997.
- Rodrigues, A.D. and Roberts, E.M., The *in vitro* interaction dexmedetomidine with human liver microsomal cytochrome P4502D6 (CYP2D6). *Drug Metab. Dispos.*, 25, 651, 1997.
- Sonnichsen, D.S., Liu, Q., Schuetz, E.G., Schuetz, J.D., Pappo, A., and Relling, M.V., Variability in human cytochrome P450 paclitaxel metabolism. *J. Pharmacol. Exp. Ther.*, 275, 566, 1995.
- Sipes, I.G. and Gandolfi, A.J., Casarett and Doull's toxicology. *The Basic Science of Poisons*, 3rd edn., Doull, J., Klassen, C.D., and Andur, M.O., Eds., MacMillan Corporation, New York, p. 88, 1992.
- Suwita, E., Nomeir, A.A., and Abou-Donia, M.B., Disposition, pharmacokinetics, and metabolism of a dermal dose of [14C]2,5-hexanedione in hens. *Drug Metab. Dispos.*, 15, 779, 1987.
- Syme, M.R., Paxton, J.W., and Keelan, J.A., Drug transfer and metabolism by the human placenta. *Clin. Pharmacokinet.*, 43, 487–514, 2004.
- Teh, L.K. and Bertilsson, L., Pharmacogenomics of CYP2D6: Molecular genetics, interethnic differences and clinical importance. *Drug Metab. Pharmacokinet.*, 27, 55–67, 2012.
- Terao, T., Hisanaga, E., Sai, Y., Tamai, I., and Tsuji A., Active secretion of drugs from the small intestinal epithelium in rats by P-glycoprotein functioning as an absorption barrier. *J. Pharm. Pharmacol.*, 48(10), 1083–1089, 1996.
- Tomkins, L.M. and Wallace, A.D., Mechanisms of cytochrome p450 induction. *J. Biochem. Mol. Toxicol.*, 21, 176–181, 2007.
- Tukey, R.H. and Strassburg, C.P., Genetic multiplicity of the human UDP-glucuronosyltransferases and regulation in the gastrointestinal tract. *Mol. Pharmacol.*, 59, 405–414, 2001.
- Uetrecht, J., Idiosyncratic drug reactions: Past, present, and future. *Chem. Res. Toxicol.*, 21, 84–92, 2008.
- Wallis, K.J., Sucralose: Features and benefits. *Food Aust.*, 45, 578–580, 1993.
- van Waterschoot, R.A.B. and Schinkel, A.H., A critical analysis of the interplay between cytochrome P450 knockout and transgenic mice. *Pharmacol. Rev.*, 63, 390–410, 2011.
- Waxman, D.J., Ko, A., and Walsh, C., Regioselectivity and stereoselectivity of androgen hydroxylation catalyzed by cytochrome P450 isozymes purified from phenobarbital-induced rat liver. *J. Biol. Chem.*, 258, 11937, 1983.
- Williams, R.T., The influence of enterohepatic circulation on toxicity of drugs. *Ann. N.Y. Acad. Sci.*, 123, 110, 1965.
- Xu, Y., Hashizume, T., Shuhart, M.C., Davis, C.L., Nelson, W.L., Sakaki, T., Kalhorn, T.F., Watkins, P.B., Schuets, E.G., and Thummel, K.E., Intestinal and hepatic CYP3A4 catalyze hydroxylation of 1 α ,25-dihydroxyvitamin D₃: Implications for drug-induced osteomalacia. *Mol. Pharmacol.*, 69, 56–65, 2006.
- Yatka, R.J., Broderick, K.B., Song, J.H., Zibell, S.E., and Urnezis, P.W., Polyvinyl acetate encapsulation of sucralose from solutions for use in chewing gum. US Patent 5169657. Assignee Wm. Wrigley Jr. Company, Chicago, IL, 1992.
- Zannoni, V.G., *Fundamental of Drug Metabolism and Drug Disposition*, Williams & Wilkins, Baltimore, MD, 1972, p. 583.
- Zerilli, A., Ratanasavanh, D., Lucas, D., Goasduff, T., Dreano, Y., Menard, C., Picart, D., and Berthou, F., Both cytochromes P450 2E1 and 3A are involved in the O-hydroxylation of *p*-nitrophenol, a catalytic activity known to be specific for P450 2E1. *Chem. Res. Toxicol.*, 10, 1205, 1997.
- Zhang, X., Liu, F., Chen, X., Zhu, X., and Uetrecht, J., Involvement of the immune system in idiosyncratic drug reactions. *Drug Metab. Pharmacokinet.*, 26, 47–59, 2011.
- Ziegler, D.M., McKee, E.M., and Poulsen, L.L., Microsomal flavoprotein catalyzed N-oxidation of acrylamides. *Drug Metab. Dispos.*, 1, 314, 1973.

18 Human Clinical Toxicology

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INTRODUCTION

Nearly 2 million human poisonings are reported to poison information centers each year; however, there are an estimated 2–3 million additional unreported exposures.¹ The purpose of this chapter is to review the epidemiological characteristics of human poisoning, clinical toxicology research designs, and general management techniques of the acutely poisoned patient. In addition, the role of the poison control centers in managing these patients and several contemporary issues in clinical toxicology are discussed.

Although several excellent texts contain valuable information on the toxicity and treatment of poisoned patients,^{2,3} the most up-to-date information on both human and animal poisonings is provided by The Micromedex® POISINDEX System from the Healthcare business of Thomson Reuters (http://thomsonreuters.com/content/healthcare/pdf/collateral/poisindex_brochure). It is a trusted, evidence-based resource that contains data on the chemical composition, toxicity, and the current medical management of >750,000 drugs, household chemicals, industrial and environmental toxins, and biologicals (including plant and animal toxins). POISINDEX also facilitates the identification of manufactured drugs by providing a description of the tablet/capsule shape, color, and the symbols imprinted on them. It also provides slang terminology, color, and shape for street drugs.

Another valuable source of information is a regional poison control center. Currently, there are ~60 regional poison control centers located throughout the United States; many have been certified by the American Association of

Poison Control Centers (AAPCC) (see Section “Role of the Poison Control Center”).

CLINICAL RESEARCH DESIGN

Predicting the effects of toxic agents in animals is critical and the effects are mandatory facts of human risk management. Basic to understanding human toxicities is the assumption that information gained from animal models can be extrapolated to analogous human situations. Other relevant information, from epidemiology studies and clinical research, is then integrated with the animal data to make regulatory decisions regarding human safety.

The principle of research design in clinical toxicology involves assessing causation in disease–exposure associations, evaluating the appropriateness of the research design, and evaluating the validity of a particular research study.

The various research designs available to study clinical problems include the randomized clinical trials (RCT), cohort studies, case–control studies, cross-sectional studies, case series, and case reports.

The advantages and disadvantages of each method are beyond the scope of this chapter and are outlined in Chapter 22 and discussed in several excellent sources on epidemiology.^{4–7}

EPIDEMIOLOGY OF POISONING

GENERAL CHARACTERISTICS

Several million poisonings occurred nationwide each year. Of the reported exposures, <0.1% results in death.

Although nonpharmaceuticals are involved in the majority of all poisonings (Table 18.1), pharmaceuticals are most frequently involved in fatalities (Table 18.2).

Ingestion is the most common route of exposure (Table 18.3). Accidental exposures account for >80% of all poisonings (Table 18.4); they are most common in children

younger than 6 years of age and in the elderly. Intentional exposures are most common in teens and adults and the most frequent cause of death in the adult population. Most accidental poisonings involved only one substance; however, the majority of intentional poisonings in adults are polydrug exposures.

TABLE 18.1
Substances Most Frequently Involved in Human Exposure 1991 and 2010

1991	% of All Exposures	2010	% of All Exposures
Cleaning substances	10.4	Analgesics	9.7
Analgesics	10.0	Cosmetics/personal care products	9.7
Cosmetics	8.3	Cleaning substances	8.4
Plants	6.1	Foreign bodies/toys/miscellaneous	5.3
Cough and cold preparations	5.7	Topicals	5.0
Bites/envenomations	4.2	Pesticides	4.0
Pesticides	3.8	Antihistamines	3.2
Topicals	3.8	Sedative/hypnotics/antipsychotics	3.1
Antimicrobials	3.5	Bites/envenomations	3.1
Foreign bodies	3.5	Vitamins	2.9
Hydrocarbons	3.5	Cough and cold preparations	2.7
Sedative/hypnotics/ antipsychotics	3.2	Antimicrobials	2.6
Chemicals	2.9	Plants	2.4
Alcohols	2.7	Cardiovascular drugs	2.3
Food poisoning	2.5	Antidepressants	2.0
Vitamins	2.2	Gastrointestinal preparations	2.0

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.2
Categories with Largest Number of Deaths
1991 and 2010

1991	2010
Analgesics	Acetaminophen combinations
Antidepressants	Acetaminophen alone
Sedative/hypnotics	Opioids
Stimulants and street drugs	Miscellaneous unknown drugs
Cardiovascular drugs	Cardiovascular drugs
Alcohols	Gases, fumes, vapors
Gases and fumes	Stimulants and street drugs
Asthma therapies	Alcohols
Chemicals	Sedative/hypnotics
Hydrocarbons	Chemicals
Cleaning substances	Acetylsalicylic acid alone
Pesticides	Cyclic antidepressants

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.3
Distribution of Route of Exposure for Human Poison
Cases: 1991 and 2010

Route	% of All Cases 1991	% of All Cases 2010
Ingestion	76.3	79.5
Dermal	7.4	6.9
Ocular	6.2	4.3
Inhalation/nasal	5.6	5.5
Bites and stings	3.7	2.5
Parenteral	0.3	0.7
Other ^a	0.3	0.4
Unknown	0.3	0.4

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

^a For 2010 includes otic (0.1%), aspiration (0.06%), vaginal (0.04%), and rectal (0.03%).

TABLE 18.4
Percent Distribution of Reason for Exposure by Age: 1991 and 2010

Reason	≤5 Years	6–12 Years	Teens ^a	Adults ^b	Unknown Child	Unknown Age	Total
Unintentional							
1991	59.5 (%)	5.1	2.2	13.6	— ^c	7.1	87.5
2010	50.3	5.4	2.8	22.4	0.2	0.3	81.4
Intentional							
1991	0.1	0.3	2.2	6.4	—	1.5	10.5
2010	0.04	0.4	3.4	10.7	0.01	0.7	15.3
Adverse reaction							
1991	0.2	0.1	0.1	0.9	—	0.4	1.6
2010	0.2	0.1	0.2	2.0	0.01	0.04	2.6
Other							
1991	—	—	—	—	—	—	—
2010	0.09	0.08	0.09	0.5	0.01	0.01	0.8
Unknown							
1991	0.0	0.0	0.0	0.2	—	0.1	0.3
2010	0.03	0.03	0.08	0.4	<0.01	0.03	0.6
Total 1991	59.8	5.5	4.5	21.1	—	9.1	100
Total 2010	50.7	6.0	6.6	36.0	0.23	1.1	100

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

^a 13–17 years for 1991; 13–19 years for 2010.

^b ≥18 years for 1991; ≥20 years for 2010.

^c Data not available.

PEDIATRIC POISONINGS

The majority of exposures involve children younger than 6 years of age. A 5 year (1985–1989) retrospective analysis of pediatric deaths was conducted to aid poison prevention and educational efforts, guide new product formulation and aversive agent use, reassess over-the-counter status for selected pharmaceuticals, and identify areas of research for the treatment of pediatric poisonings.⁸ A hazard factor was devised to assess the risk of each agent to produce a major (residual disability) or life-threatening outcome when a child is involved in an overdose. This factor indicates a substance's relative pediatric hazard by evaluating its packaging, accessibility (as a reflection of common storage practices in the home), availability (as a reflection of marketing), formulation, and closure types. The hazard score ranking allows for a comparison among categories.

Of the 3.8 million pediatric exposures reported between 1985 and 1989, 2117 patients experienced a major outcome and 111 fatalities occurred. Table 18.5 shows the substance categories implicated in pediatric exposure calls, the total number of pediatric exposures that occurred in each category, the number of major effects, the number of fatal exposures, and the hazard factor. Tables 18.6 and 18.7 address unintentional pediatric ingestion fatalities reported to the AAPCC in 1983 through 1990. Iron supplements emerge as the single-most frequent cause of unintentional death in pediatrics, representing >30% of all deaths.

The ingestion hazards parallel the substances required to have child-resistant closures by the Poison Prevention

Packaging Act. This demonstrates, however, that the requirement of child-resistant closures does not render a product “childproof.” In 1989, King and Palmisano performed an epidemiological study to identify the risk factors responsible for the ineffectiveness of child-resistant closures. Although the Poison Prevention Packaging Act of 1970 resulted in a 65% decline in the ingestion of products packaged in child-resistant containers, ingestion of prescription drugs by children declined by only 36%. Reasons for these data include

1. Availability of nonchild-resistant packaging upon consumer request (i.e., consumer noncompliance)
2. Misuse of child-resistant closures by the consumer in the home
3. Transferring medicines from child-resistant packages to unsafe containers, or using no container at all
4. Unsafe storage practices, such as leaving containers within easy reach
5. Violations of the ACT by the dispensing pharmacist, physician, or Health Care Facility*

Pharmacies and healthcare facilities have also been shown to be noncompliant with the packaging act of 1970.

Table 18.8 demonstrates that 2 year olds have accounted for the majority of ingestions of prescription drugs.⁹ Table 18.9 demonstrates that in >75% of cases surveyed,

* Reprinted with permission from King and Palmisano.⁹

nonchild-resistant packages or no containers were involved in the ingestion.⁹ Table 18.10 demonstrates that the owner of each prescription was able to be identified in 80% of the cases surveyed. According to the survey by King and Palmisano, although parents' prescriptions accounted for 53.6% of the ingestions, nearly 30% involved grandparents' medications.⁹ Noncompliance with child-resistant packaging was a major reason for the exposure as indicated in Table 18.11.⁹

Other barriers to pediatric poisonings include the use of warning stickers designed to deter children from getting into containers. However, studies have failed to demonstrate any benefit from their use and, in fact, the warning stickers may attract children who otherwise would have ignored the product.¹⁰ Woolf and Lovejoy¹⁰ discuss the epidemiology of drug overdose in children and the determinants that result in a high risk of drug poisoning in this group.

TABLE 18.5
Reported Poison Exposures in Children Younger than 6 Years of Age

Substance Categories and Subcategories ^a	Total Pediatric Exposures (1985–1989)	No. of Major Effects	No. of Deaths	Hazard Factor ^b
Nonpharmaceutical exposures				
Adhesives/glues	37,986	15	0	0.7
Alcohols	80,443	46	5	1.0
Ethanol (beverage)	2,622	11	2	8.0*
Arts/crafts/office supplies	80,294	3	0	0.1
Auto/aircraft/boat products	12,019	11	4	2.0
Ethylene glycol	2,321	4	1	3.5*
Methanol	1,883	3	3	5.1*
Batteries	12,753	7	0	0.9
Bites/envenomations	48,821	100	0	3.3*
Copperhead	44	4	0	146.4*
Rattlesnake	125	19	0	244.8*
Unknown snake	651	10	0	24.7*
Other/unknown reptile	682	3	0	7.1*
Scorpion	1,585	43	0	43.7*
Black widow spider	1,240	4	0	5.2*
Brown recluse spider	455	2	0	7.1*
Building products	13,721	2	0	0.2
Chemicals	87,463	72	3	1.4*
Acid: hydrochloric	784	3	0	6.2*
Alkali	10,267	24	0	3.8*
Dioxin	11	1	0	146.4*
Ethylene glycol	1,002	6	0	9.6*
Strychnine	32	1	0	50.3*
Cleaning substances	386,052	205	4	0.9
Acid: drain cleaner	208	6	0	46.5*
Acid: industrial cleaner	438	3	0	11.0*
Alkali: drain cleaner	1,474	19	1	21.9*
Alkali: industrial cleaner	938	17	0	29.2*
Alkali: oven cleaner	4,619	10	0	3.5*
Oven cleaner: other/unknown	224	4	0	28.8*
Cosmetics/personal care	395,985	57	3	0.2
Deodorizers (nonpersonal)	39,408	1	0	0.0
Dyes	10,369	0	0	0.0
Essential oils	6,557	5	0	1.2
Fertilizers	23,581	1	0	0.1
Fire extinguishers	860	0	0	0.0
Food products/poisoning	49,500	7	0	0.2
Foreign bodies/toys	163,722	21	0	0.2
Fumes/gases/vapors	8,436	55	21	14.5*
Carbon monoxide	3,103	42	18	31.1*
Chlorine gas	2,208	6	0	4.4*
Hydrogen sulfide	228	2	0	14.1*

TABLE 18.5 (continued)
Reported Poison Exposures in Children Younger than 6 Years of Age

Substance Categories and Subcategories ^a	Total Pediatric Exposures (1985–1989)	No. of Major Effects	No. of Deaths	Hazard Factor ^b
Methane and natural gas	700	3	1	9.2*
Gas: other	1,138	1	2	4.2*
Fungicides (nonmedicinal)	2,714	1	0	0.6
Heavy metals	11,926	14	0	1.9*
Other	1,094	4	0	5.9*
Unknown	39	1	0	41.3*
Herbicides	6,488	3	0	0.7
Paraquat	66	1	0	24.4*
Hydrocarbons	129,024	168	5	2.2*
Kerosene	10,751	51	2	7.9*
Lighter fluid/naphtha	8,865	24	0	4.4*
Mineral seal oil	6,564	13	0	3.2*
Insecticides/pesticides	100,105	122	6	2.1*
Chlorinated hydrocarbon alone	9,694	29	0	4.8*
Organophosphate alone	16,560	56	2	5.6*
Organophosphate with other pesticide	1,806	4	0	3.6*
Rotenone	284	2	0	11.3*
Lacrimators	4,779	1	0	0.3
Matches/fireworks/explosive	11,655	1	0	0.1
Moth repellants	19,548	6	0	0.5
Mushrooms	32,724	9	0	0.4
Paints/stripping agents	47,114	10	0	0.3
Photographic products	2,688	1	0	0.6
Plants	375,649	33	1	0.1
Polishes/waxes	12,249	4	0	0.5
Rodenticides	41,261	2	1	0.1
Sporting equipment	2,134	0	4	3.0*
Gun bluing compound	100	0	3	48.3*
Swimming pool/aquarium	8,067	1	0	0.2
Tobacco products	36,742	14	0	0.6
Unknown nondrug substance	20,318	14	0	1.1
Pharmaceutical exposures				
Analgesics	325,539	119	8	0.6
Acetaminophen with propoxyphene	2,171	6	0	4.5*
Aspirin: unknown formulation	10,002	18	1	3.1*
Methadone	127	4	2	76.1*
Morphine	164	3	0	29.5*
Propoxyphene	514	1	1	6.3*
Other/unknown narcotic	732	4	0	8.8*
Anesthetics	14,025	17	4	2.4
Anticholinergic	6,516	13	0	3.2*
Anticoagulants	1,860	1	0	0.9
Anticonvulsants	9,198	106	4	19.3*
Carbamazepine	4,113	81	2	32.5*
Phenytoin	3,619	19	2	9.3*
Valproic acid	1,197	5	0	6.7*
Other anticonvulsant	54	1	0	29.8*
Antidepressants	12,003	125	7	17.7*
Amitriptyline	2,897	39	2	22.8*
Amoxapine	200	4	0	32.2*
Desipramine	935	13	3	27.6*
Doxepin	887	8	0	14.5*

(continued)

TABLE 18.5 (continued)
Reported Poison Exposures in Children Younger than 6 Years of Age

Substance Categories and Subcategories ^a	Total Pediatric Exposures (1985–1989)	No. of Major Effects	No. of Deaths	Hazard Factor ^b
Imipramine	2,503	29	2	20.0*
Maprotiline	209	3	0	23.1*
Nortriptyline	347	2	0	9.3*
Other cyclic antidepressant	179	2	0	18.0*
Unknown cyclic antidepressant	142	5	0	56.7*
Cyclic antidepressant with benzodiazepine	288	2	0	11.2*
Cyclic antidepressant with phenothiazine	832	10	0	19.4*
Lithium	1,054	7	0	10.7*
Antihistamines	38,390	20	4	1.0
Antimicrobials	122,686	28	3	0.4
Antimalarials	204	2	2	31.6*
Isoniazid	219	3	0	22.1*
Rifampin	84	2	0	38.4*
Antineoplastics	570	0	1	2.8
Asthma therapies	20,502	43	3	3.6*
Aminophylline	8,622	38	3	7.7*
Cardiovascular drugs	37,385	182	7	8.1*
Antiarrhythmics	1,203	3	0	4.0*
Antihypertensives	8,099	139	0	27.6*
Cardiac glycosides	3,846	24	2	10.9*
Nitroprusside	47	1	1	68.5*
Cough/cold preparations	249,038	72	4	0.5
Diagnostic agents	697	1	0	2.3
Diuretics	11,175	8	0	1.2
Electrolytes/minerals	50,751	57	8	2.1*
Iron	11,234	52	7	8.5*
Eye/ear/nose/throat preparations	32,805	17	0	0.8
Glaucoma medications	74	1	0	21.8*
Gastrointestinal preparations	99,636	48	3	0.8
Antidiarrheals: diphenoxylate/atropine	2,500	18	1	12.2*
Hormones and antagonists	6,357	20	1	0.5*
Insulin	217	2	1	22.3*
Oral hypoglycemics	2,609	11	0	6.8*
Miscellaneous drugs	17,650	10	2	1.1
Neuromuscular blocking agents	8	2	0	402.7*
Muscle relaxants	3,165	7	0	3.6*
Methocarbamol	341	2	0	9.4*
Other	1,012	3	0	4.8*
Sedative/hypnotics/antipsychotics	33,048	153	2	7.6*
Barbiturates: long acting	4,475	37	0	13.3*
Barbiturates: short acting	1,051	4	0	6.1*
Chloral hydrate	443	18	0	65.5*
Ethchlorvynol	81	1	0	19.9*
Glutethimide	30	2	0	107.4*
Methaqualone	66	1	0	24.4*
Phenothiazines	7,451	57	2	12.8*
Other	330	2	0	9.8*
Serums, toxoids, vaccines	448	0	0	0.0
Stimulants/street drugs	21,260	84	1	6.4*
Amphetamines	6,409	18	0	4.5*
Cocaine	546	20	0	59.0*
Lysergic acid diethylamide	117	2	0	27.5*
Marijuana	694	3	0	7.0*
Mescaline/peyote	325	2	0	9.9*
Phencyclidine	177	30	0	273.0*

TABLE 18.5 (continued)
Reported Poison Exposures in Children Younger than 6 Years of Age

Substance Categories and Subcategories ^a	Total Pediatric Exposures (1985–1989)	No. of Major Effects	No. of Deaths	Hazard Factor ^b
Topicals	175,378	55	2	0.5
Silver nitrate	44	1	0	36.6*
Miscellaneous veterinary	4,630	0	0	0.0
Vitamins	145,872	33	1	0.4
Unknown drugs	21,020	38	0	2.9*
Total	3,852,618	2,270	122	1.0

Source: Litovitz, T. and Manoguerra, A., *Pediatrics*, 89, 999, 1992. Reprinted with permission.

^a Subcategories with hazard factors ≥ 3 and statistical significance are listed under each substance category.

^b See text for explanation.

* $p < 0.05$, Fisher's exact test comparing each individual category (or subcategory) with all other cases.

TABLE 18.6
Pediatric Pharmaceutical
Ingestion Fatalities: 1983–1990

Substances Ingested	N	% Total
Anticonvulsants	3	5.7
Antidepressants	10	18.9
Cardiovascular drugs	8	13.2
Iron supplements	16	30.2
Salicylates	6	11.3
Miscellaneous	11	20.7

Source: Litovitz, T. and Manoguerra, A., *Pediatrics*, 89, 999, 1992. Modified with permission.

TABLE 18.7
Pediatric Nonpharmaceutical
Ingestion Fatalities 1983–1990

Substances	N	%
Pesticides	12	27.3
Hydrocarbons	12	27.3
Alcohols and glycols	7	15.9
Gun bluing	4	9.1
Cleaning substances	3	6.8
Chemicals	3	6.8
Cosmetics and personal care products	2	4.5
Plants	1	2.3

Source: Modified from Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992. With permission.

TABLE 18.8
Ingestions of Solid Prescription
Drugs by Age of Victim

Age Categories (Months)	No.	(%)
Infant (<12 months)	1	(0.1)
1 (12–23)	205	(24.2)
2 (24–35)	456	(53.7)
3 (36–47)	124	(14.6)
4 (48–59)	37	(4.4)
5 (60–71)	25	(2.9)
Unknown	1	(0.1)
Total	849	(100.0)

Source: Reprinted from King, W. and Palmisano, P., *S. Med. J.*, 82, 1468, 1989. With permission.

TABLE 18.9
Ingestions of Solid Prescription
Drugs by Container Type

Container Type	No. ^a	(%)
Child-resistant	159	(24.1)
Nonchild-resistant	268	(40.5)
No container	214	(32.4)
Other	20	(3.0)
Total	661	(100.0)

Source: Reprinted from King, W. and Palmisano, P., *S. Med. J.*, 82, 1468, 1989. With permission.

^a Number reporting container type.

TABLE 18.10
Owner of Medication Ingested

Owner	No.	(%)	Cumulative %
Mother	294	(43.3)	43.3
Father	70	(10.3)	53.6
Grandmother	143	(21.0)	74.6
Grandfather	51	(7.5)	82.1
Sibling	10	(1.5)	83.6
Self	4	(0.6)	84.2
Dog	4	(0.6)	84.8
Other (neighbor, relative)	103	(15.2)	100.0
Total	679	(100.0)	

Source: Reprinted from King, W. and Palmisano, P., *S. Med. J.*, 82, 1468, 1989. With permission.

TABLE 18.11
Container Type and Ownership of Medication^a

	Father	Mother	Grandfather	Grandmother	Total
Child-resistant	13	82	7	27	129
No container	23	75	13	40	151
Nonchild-resistant	25	79	25	56	185
Total (%)	61 (13.1%)	236 (50.8%)	45 (9.7%)	123 (26.4%)	465
Total noncompliance (%)	48 (78.6%)	154 (65.3%)	38 (84.4%)	96 (78.0%)	
Parent's noncompliance	202 (68.0%)				
Grandparent's noncompliance			134 (79.8%)		

Source: Reprinted from King, W. and Palmisano, P., *S. Med. J.*, 82, 1468, 1989. With permission.

^a Excludes other owners of medication (i.e., neighbor, aunt, sibling, other).

ADOLESCENT TOXIC EXPOSURES

Intentional poisoning in adolescents is one of the 10 leading causes of death and potentially productive years of life lost in the United States.¹¹ Alcohol use and abuse plays a large role in fatal injuries in this age group.

Drug-related fatalities by drug class among adolescents 11–17 years old reported to the AAPCC from 1989 to 1991 are listed in Table 18.12. Of 764 fatalities reported in 1991, 52 (6.8%) were in the adolescent age group; 92% were 13–17 years old.

TABLE 18.12
Drug-Related Fatalities by Drug Class among Adolescents
11–17 Years Old Reported to the American Association
of Poison Control Centers

Drug Class	1989	1990	1991	Total
Cyclic antidepressants	13	7	11	31
Amitriptyline	1	3 ^a	1	
Desipramine	4	2	7 ^b	
Doxepine	1 ^c	0	2	
Imipramine	5 ^d	1	0	
Maprotiline	1	0	0	
Nortriptyline	1 ^e	1	1	
Calcium channel blockers	2	2	3	7
Nifedipine	0	0	1	
Verapamil	2 ^f	2 ^g	2 ^h	
Salicylates	1	0	5	6
Theophylline	3 ⁱ	1	1	5
Propranolol	3	2 ^j	1	5
Methamphetamine	1	0	0	1
Cocaine	0	0	1	1
Street drug (unknown type)	1	0	0	1
Paracetamol (acetaminophen)	1	0	2 ^k	3
Carbamazepine	1 ^l	0	0	1
Haloperidol	0	1	0	1
Glipizide + cyclobenzaprine	0	1	0	1
Isoniazid	0	0	1	1
Lidocaine (lignocaine)	0	1	0	1
Methocarbamol	0	0	1	1
Chlorpromazine	0	0	1	1
Temazepam	0	0	1	1
Colchicine/allopurinol/ibuprofen	0	0	1	1
Amfebutamone (bupropion) + lithium	0	1	0	1
Phenylpropanolamine/chlorphenamine	0	1	0	1
Paracetamol + doxylamine + detromethorphan + pseudoephedrine	0	1	0	1
Total	26	18	29	72

Source: Reprinted from Woolf, A. and Lovejoy, F., *Drug Safety*, 9, 291, 1993. With permission.

^a Ethanol also present in 1 case, thioridazine + alprazolam in another, methyl dopa + perphenazine in the third case.

^b Salicylate also present in 1 case.

^c Verapamil + piroxicam also present.

^d Tranlycypromine also present.

^e Mesoridazine also present.

^f Digoxin present in 1 case, meclizine present in the other.

^g Propranolol also present in one case.

^h Naproxen + propranolol also present in 1 case.

ⁱ Amoxicillin + cefalexin present in 1 case, ephedrine in another.

^j Atenolol also present.

^k Dextropropoxyphene also present in 1 case.

^l Ampicillin + erythromycin also present.

POISONING IN THE ELDERLY

Poisoning in the elderly is a continuing public health problem. Accidental poisoning, due to dementia and confusion, improper use or storage of a product, and therapeutic errors account for the most exposures of patients >64 years of age.¹² A smaller percentage (~10%) are intentional with suicidal intent. The mortality rate from poisoning is much higher in the

elderly than in other age groups. Of the total fatalities reported in 1991 and 2010, ~20% were ≥60 years old (Table 18.13).

Woolf et al.¹³ analyzed poisoning-related hospitalization and mortality rates among older adults in Massachusetts from 1983 to 1985. The poisoning hospitalization rates and poisoning-related death rates are listed in Tables 18.14 and 18.15. Table 18.16 lists intentionality and agent of poisoning deaths for 152 of the 275 total deaths.

TABLE 18.13
Distribution of Age for Fatalities: 1991 and 2010

Age (Years)	No. of Fatalities 1991	No. of Fatalities 2010	Percent 1991	Percent 2010	Cumulative Percent 1991	Cumulative Percent 2010
<1	5	3	0.7	0.3	0.7	0.3
1	17	9	2.2	0.8	2.9	1.1
2	13	6	1.7	0.5	4.6	1.6
3	5	6	0.7	0.5	5.2	2.1
4	1	5	0.1	0.4	5.4	2.5
5	3	3	0.4	0.3	5.8	2.8
6–12	4	3	0.5	0.3	6.3	3.1
13–19	64	56	8.4	4.9	14.7	7.9
20–29	119	172	15.6	15.0	30.2	23.0
30–39	179	184	23.4	16.1	53.7	39.0
40–49	114	237	14.9	20.7	68.6	59.7
50–59	67	224	8.8	19.6	77.4	79.2
60–69	55	119	7.2	10.4	84.6	89.6
70–79	53	54	6.9	4.7	91.5	94.3
80–89	33	50	4.3	4.4	95.8	98.7
90–99	11	9	1.4	0.8	97.3	99.5
Unknown adult	21	3	2.8	0.3	100	99.7
Unknown age		3		0.3		100
Total	764	1,146	100	100		

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.14
Annualized Poisoning Hospitalization Rates and Relative Risks, Massachusetts 1983–1985

Age Groups (Years)	Males		Females	
	(Hosp/10 ⁵ /Year)	RR ^a (95% CI)	(Hosp/10 ⁵ /year)	RR ^a (95% CI)
60–64	44	0.47 (0.40, 0.55)	58	0.47 (0.42, 0.53)
65–69	57	0.61 (0.52, 0.70)	64	0.52 (0.46, 0.58)
70–74	58	0.62 (0.52, 0.73)	74	0.60 (0.52, 0.67)
75–79	64	0.68 (0.56, 0.83)	91	0.73 (0.65, 0.83)
80–84	116	1.23 (1.01, 1.50)	102	0.82 (0.72, 0.95)
85	112	1.19 (0.94, 1.51)	96	0.77 (0.67, 0.89)
State average (all ages)	89		114	
State average (persons less than 60 years old)	94		124	

Source: Reprinted from Woolf, A. et al., *Am. J. Public Health*, 80, 867, 1990. With permission.

^a RR, Relative risk (age-specific rate/state average rate, <60 year). 95% confidence intervals in parentheses.

TABLE 18.15
Annualized Poisoning-Related Death Rates and Relative Risks, Massachusetts
1983–1985

Age Group (Years)	Males		Females	
	(Deaths/10 ⁵ /Year)	RR ^a (95% CI)	(Deaths/10 ⁵ /Year)	RR ^a (95% CI)
60–64	8.34	1.04 (0.73, 1.48)	6.62	1.74 (1.20, 2.22)
65–69	7.45	0.93 (0.62, 1.40)	7.04	1.85 (1.26, 2.70)
70–74	14.43	1.80 (1.27, 2.55)	5.68	1.49 (0.95, 2.34)
75–79	13.07	1.63 (1.04, 2.55)	7.79	2.04 (1.32, 3.15)
80–84	14.00	1.75 (0.99, 3.09)	6.07	1.59 (0.89, 2.84)
85+	20.54	2.56 (1.48, 4.44)	13.07	3.43 (2.28, 5.16)
State average (all ages)	9.37		5.16	
State average (persons <60 years old)	8.02		3.81	

Source: Reprinted from Woolf, A. et al., *Am. J. Public Health*, 80, 867, 1990. With permission.

^a RR, Relative risk (age-specific rate/state average rate, <80 year). 95% confidence intervals in parentheses.

TABLE 18.16
Intentionality and Agent of Poisoning Deaths in Massachusetts Adults 60 Years
and Older, 1983–1985^a

Circumstance/intentionality	Sex	
	Male	Female
	<i>N</i> = 78	<i>N</i> = 78
Accidental/unintentional		
Drugs	20	26
Carbon monoxide	5	5
Isopropanol	1	0
Benzene	1	0
Subtotal	27	31
Indeterminate		
Drugs	3	9
Suicide/intentional		
Drugs	9	32
Carbon monoxide	31	5
Corrosive	1	0
Carbon tetrachloride	1	0
Other agents	2	0
Subtotal	44	38

Source: Reprinted from Woolf, A. et al., *Am. J. Public Health*, 80, 867, 1990. With permission.

^a This table includes only those deaths for which E-codes were available (152 out of 275 total deaths).

MANAGEMENT TRENDS

Table 18.17 outlines the frequency of initial decontamination procedures, measures used to enhance toxin elimination, and antidotes administered to patients reported to the 1991 and 2010 AAPCC database. The use of Syrup of Ipecac as an initial method of gastrointestinal decontamination has

drastically declined in the emergency department setting. When used, it has been most often administered to the pediatric patient (Table 18.18). Table 18.19 demonstrates a continued decline from 1985 to 2010 in the use of emesis induced by Syrup of Ipecac and a relatively similar use of activated charcoal administration in the emergency department.

TABLE 18.17
Therapy Provided in Human Exposure Cases

Therapy	1991	2010
Initial decontamination		
Dilution/irrigation/washing	1,020,264	929,574
Activated charcoal	129,203	74,431
Cathartic	107,556	17,842
Ipecac syrup	95,124	360
Gastric lavage	58,677	4,883
Other emetic	4,239	11,264
Measures to enhance elimination		
Alkalinization (with or without diuresis)	7,092	10,260
Hemodialysis	692	2,298
Forced diuresis	500	None reported
Hemoperfusion (charcoal)	124	23 (not specified)
Exchange transfusion	106	None reported
Acidification (with or without diuresis)	99	None reported
Hemoperfusion (resin)	38	See earlier for charcoal
Peritoneal dialysis	11	None reported
Specific antidote administration		
Naloxone	7,136	18,621
N-Acetylcysteine (oral)	7,075	8,362
Atropine	923	1,254
Deferoxamine	901	61
Antivenim	579	346 (includes antitoxin)
Ethanol	540	111
Hydroxocobalamin	384	53
N-Acetylcysteine (IV)	250	16,961
Pralidoxime (2-PAM)	248	77
Fab fragments	241	2,205
Pyridoxine	227	491
Physostigmine	226	245
Dimercaprol (BAL)	146	38
Methylene blue	117	100
Cyanide antidote kit (sodium nitrite, sodium thiosulfate, amylnitrite)	99	110
Ethylenediaminetetraacetic acid (EDTA)	94	61
Penicillamine	86	4
Total	1,442,997	1,100,035

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.18
Ipecac Administration by Age

Age (Years)	Number of Cases 1991	Percent of Cases 1991	Number of Cases 2010	Percent of Cases 2010
≤5	73,133	76.9	163	45.3
6–12	2,156	2.3	24	6.7
13–17	5,587	5.9		
13–19			55	15.3
>17	13,713	14.4		
>19			109	30.3
Unknown	535	0.6	9	2.5
Total	95,124	100	360	100

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.19
Decontamination Trends

Year	Ipecac Administered (% of Human Exposures Reported)	Activated Charcoal Administered (% of Human Exposures Reported)
1985	15.0	4.6
1990	6.0	6.6
1995	2.3	7.7
2000	0.8	6.7
2005	0.1	5.1
2010	0.02	3.1

Source: Modified from Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

ROLE OF THE TOXICOLOGY LABORATORY

Toxicological testing or a “Tox Screen” of blood, urine, and gastric contents is frequently ordered for patients with suspected drug overdoses in the emergency department. Because 10–15 drugs account for >90% of all drug overdoses, most laboratories limit the number of drugs tested to the common drugs of abuse and other agents, such as over-the-counter analgesics (Table 18.20). Osterloh¹⁴ reviewed and evaluated the circumstances, types of toxicological testing, utility, reliability, and application of the laboratory tests in the emergency evaluation of the overdosed patient.

Initial workup of an intoxicated patient always includes a history and physical examination as an assessment of their condition. During this initial evaluation, a preliminary toxicological diagnosis may be made based on a constellation of signs and symptoms (Tables 18.21 and 18.22). Drug and

nondrug tests may be useful in the diagnosis. Examples of these tests are listed in Table 18.23. A comparison of toxicological methods is outlined in Table 18.24.

A toxicology screen uses various methodologies to identify the drugs most frequently used or abused by the poisoned patient. The screens are usually focused on identifying the common drugs of abuse. Drug quantitation in serum is used to monitor the course of the patient, to diagnose whether toxicity is occurring, but not yet clinically apparent, to establish a prognosis, and to determine whether extracorporeal methods of toxin elimination will be necessary. In the emergency setting, there are relatively few toxins that require quantitation to have an impact on patient management. The toxins that require quantitation in emergency toxicology and the potential interferences in assays are listed in Table 18.25.

TABLE 18.20
Common Drugs Included on Most Toxicology Screens

Alcohols—Ethanol, methanol, isopropanol, acetone
Barbiturates/sedatives—Amobarbital, secobarbital, pentobarbital, butalbital, butabarbital, phenobarbital, glutethimide, ethchlorvynol, methaqualone
Antiepileptics—Phenytoin, carbamazepine, primadone, phenobarbital
Benzodiazepines—Chlordiazepoxide, diazepam, alprazolam, temazepam
Antihistamines—Diphenhydramine, chlorpheniramine, brompheniramine, tripeleminamine, trihexiphenidyl, doxylamine, pyrilamine
Antidepressants—Amitriptyline, nortriptyline, doxepin, imipramine, desipramine, trazodone, amoxapine, maprotiline
Antipsychotics—Trifluoperazine, perphenazine, prochlorperazine, chlorpromazine
Stimulants—Amphetamine, methamphetamine, phenylpropanolamine, ephedrine, MDA, MDMA (other phenylethylamines), cocaine, phencyclidine
Narcotics analgesics—Heroin, morphine, codeine, oxycodone, hydrocodone, hydromorphone, meperidine, pentazocine, propoxyphene, methadone
Other analgesics—Salicylates, acetaminophen
Cardiovascular drugs—Lidocaine, propranolol, metoprolol, quinidine, procainamide, verapamil
Others—Theophylline, caffeine, nicotine, oral hypoglycemics, strychnine

Source: Reprinted from Osterloh, J., *Emerg. Med. Clin. N. Am.*, 8, 693, 1990. With permission.

TABLE 18.21
Some Common Clinical Presentations and Differential Diagnoses in Overdose

Presentation	Toxicological Causes	Other Medical Examples
Asymptomatic with history	Almost any drug	Not applicable
Gastrointestinal complaints	Salicylate, theophylline, iron, colchicine quinidine, almost any drug	Food poisoning, allergy, ulcer, pancreatitis, obstruction, gallstones, genitourinary
Coma	Narcotics, sedatives, antipsychotics, alcohol, tricyclics, long-lasting benzodiazepines	Infectious and metabolic encephalopathy, trauma, anoxia, cerebrovascular accident, brain death
Seizures	Theophylline, tricyclics, isoniazid, stimulants, camphor, carbon monoxide, hypoglycemic agents, alcohol withdrawal	Idiopathic, arteriovenous malformation, tumor, trauma, hypoxia, febrile, inborn errors
Psychosis and altered mental status	Anticholinergics, stimulants, withdrawal	Psychiatric, infection, metabolic/inborn errors
Acidosis	Salicylate, ethanol, methanol, ethylene glycol, cyanide drugs causing seizures	Shock, diabetes, uremia, lactic acidosis

(continued)

TABLE 18.21 (continued)
Some Common Clinical Presentations and Differential Diagnoses in Overdose

Presentation	Toxicological Causes	Other Medical Examples
Respiratory depression (usually with coma)	Narcotics, sedatives, benzodiazepines	Cerebrovascular accident, metabolic coma, tumor
Pulmonary edema	Salicylates, narcotics, iron, paraquat (initially)	Heart failure, disseminated intravascular coagulation
Arrhythmias	Tricyclics, quinidine, anticholinergics, β -blockers, digoxin, lithium, antipsychotics, organophosphates	Atherosclerotic heart disease
Hypotension	Narcotics, sedatives, tricyclics, antipsychotics, β -blockers, theophylline, iron	Heart failure, shock, hypovolemia, disseminated intravascular coagulation
Hypertension	Cocaine, amphetamines, cyanide, nicotine, clonidine (initially)	Essential, pheochromocytoma, carcinoid, hyperrenin states, renal failure
Ataxia	Antiepileptics, barbiturates, alcohol, lithium, organomercury	Cerebellar degeneration

Source: Reprinted from Osterloh, J., *Emerg. Med. Clin. N. Am.*, 8, 693, 1990. With permission.

TABLE 18.22
Toxicological Syndromes by Class of Drugs

Narcotics

Heroin, morphine, codeine, oxycodone, hydromorphone, hydrocodenone, propoxyphene, pentazocine, meperidine, diphenoxylate, fentanyl and derivatives, buprenorphine, methadone

CNS depression (somnolent \rightarrow coma)	If BP decreases, pulse does not increase
Slowed respiratory rate	Pinpoint pupils
T $^{\circ}$ normal or low	DTR usually decrease

Alcohols—barbiturates

Ethanol, methanol, isopropanol, ethylene glycol, amo-, pento-, seco-, buta-, phenobarbital, butalbital, glutethimide, methaqualone, ethchlorvynol, phenytoin

CNS depression (stuporous \rightarrow coma)	DTR decreases
Ataxia	Metabolic acidosis with alcohols and ethylene glycol except isopropanol
T $^{\circ}$ usually decreases	If BP decreases, pulse may increase

Anticholinergics

Atropine, scopolamine, antihistamines, phenothiazines, tricyclics, quinidine, amantadine, jimson weed, mushrooms

Delirious	Decreased bowel sounds
Increased pulse, increased T $^{\circ}$	Urinary retention
Skin flushed, warm, pink	Blurred vision
Dry (no sweating)	Arrhythmias, prolonged QT

Stimulants

Cocaine, amphetamines, and derivatives (e.g., ice, MDA, MDMA, DOB), phencyclidine, lysergic acid, psilocybin

Acute psychosis (nonreality)	Increased muscle tone/activity
Increased pulse, increased BP, increased T $^{\circ}$	Dilated pupils
Increased respiratory rate	Sweating
Agitation	Seizures

TABLE 18.22 (continued)
Toxicological Syndromes by Class of Drugs

Antidepressants

Anticholinergic syndrome	Sinus tachycardia (early)
Hypotension	Supraventricular tachycardia (early)
Coma	Widened QRS, QT
Seizures	Ventricular arrhythmias

Benzodiazepines

CNS depression	BP, pulse, T° not greatly affected
Respiratory depression	DTR intact

Phenothiazines

Decreased BP, decreased T°	Anticholinergic syndrome (see earlier)
Rigidity, dystonias, torticollis	Seizures
Pinpoint pupils	

Salicylates

Abdominal pain	Shock
Respiratory alkalosis (early)	Diaphoresis
Metabolic acidosis	Hypoglycemia

Theophylline

Tachycardia	Hypotension
Hypokalemia	Seizures

Iron

Abdominal pain	Acidosis
GI bleeding	Renal failure
Hypotension	Cardiovascular collapse
Hypovolemia	

Lithium

Tremor	Hyperreflexia
Chorea	Rigidity
Abdominal pain	Seizures

Isoniazid

Metabolic acidosis	Hepatitis
Seizures	

Oral hypoglycemics

Hypoglycemia	Diaphoresis
Coma	

Acetaminophen

Liver necrosis

β-Blockers

Bradycardia	Hypotension with slowed cardiac conduction
-------------	--

Hyperglycemia

Source: Reprinted from Osterloh, J., *Emerg. Med. Clin. N. Am.*, 8, 693, 1990. With permission.

Abbreviations: CNS, central nervous system; BP, blood pressure; DTR, deep tendon reflexes; GI, gastrointestinal; QRS, QT, electrocardiogram parameters; MDA, methylenedioxy amphetamine; MDMA, 3,4-methylenedioxy methamphetamine; DOB, 4-bromo-2,5-dimethoxyamphetamine.

TABLE 18.23
Useful Laboratory Tests in Toxicological Diagnosis

Classes of Drugs	Clinical and Laboratory Tests	Toxicological Procedures for Identification
Narcotics	IV Narcan, ABGs	IA, TLC, GC, GC-MS
Alcohols	Breath odor, osmolar gap	GC, EZ, IA
Sedatives	Calories, ABGs	IA, TLC, HPLC, GC, GC-MS
Anticholinergics	EKG, CPK, K	TLC, GC, HPLC, GC-MS
Stimulants	CPK, K	IA, TLC, GC, GC-MS
Antipsychotics	EKG, ABGs	Spot, TLC, GC, HPLC, GC-MS
Tricyclics	EKG, ABGs	Spot, IA, TLC, CC, HPLC, CC-MS
β-Blockers	EKG	TLC, GC, HPLC
Oral hypoglycemics	Serum glucose	TLC, HPLC, GC
Specific toxins		
Salicylates ^a	Anion gap, HCO ₃ gap	Spot, SM, IA, HPLC, GC
Theophylline ^a	K, glucose	IA, HPLC, SM
Acetaminophen ^a	ALT, AST	TLC, IA, SM, HPLC
Methanol ^a	Anion and osmolar gaps	GLC
Ethylene glycol ^a	Anion and osmolar gaps	GLC
Lithium ^a	Serum creatinine	Flame, ISE
Iron ^a	WBC, serum glucose, serum iron	SM
Carbon monoxide	O ₂ saturation gap	Co-oximeter, SM
Cyanide	A-V O ₂ difference	SM
Nitrites	Brown blood	Co-oximeter, SM

Source: Modified from Osterloh, J., *Emerg. Med. Clin. N. Am.*, 8, 693, 1990. With permission.

Abbreviations: EZ, enzymatic; SM, spectrometric; spot, chemical test; ISE, ion-selective electrode; flame, flame emission spectrometry; ABGs, arterial blood gases; K, potassium; CPK, creatinine phosphokinase; HCO₃, bicarbonate; IA, immunoassays; TLC, thin layer chromatography; GC, gas chromatography; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; EKG, electrocardiogram; WBC, white blood cell count; AST, ALT, liver enzyme tests; A-V, arteriovenous.

^a Require quantitation for effective therapy. Concentration-effect relationships are known.

TABLE 18.24
Comparison of Generic Toxicological Methods

Method	Specificity	Sensitivity	Multidrug Drug Detection Possible	Quantitative Ability	Turnaround Time (HR)	Labor Intensive	Technical Expertise
Chemical spot	+	+	No	No	<0.5	+	0
Spectrometric	+	+	No	Yes	<2	++	++
IA	++	++	Some	Some	<1	+	+
TLC	++	+	Yes	No	2-4	+++	+++
GC	++	++	Yes	Yes	<4	++	++
HPLC	++	++	Yes	Yes	<4	++	++
GC-MS	+++	+++	Yes	Yes	<8	+++	++++

Source: Reprinted from Osterloh, J., *Emerg. Med. Clin. N. Am.*, 8, 693, 1990. With permission.

Note: Costs in 1990, current costs will be higher.

Abbreviations: IA, immunoassays; TLC, thin layer chromatography; GC, gas chromatography; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry.

TABLE 18.25
Potential Interferences for Quantitative Serum Drug Tests Used in Emergency Toxicology^a

Drug	Generic Method	Interferences
Salicylate	SC	Phenothiazines, ^b ketosis, ^b acetaminophen, diflunisal, salicylamide, phenylketones, around 10% inc due to cross-reaction with accumulated salicylate metabolites in RF
	GC ^c	Methylsalicylate, eucalyptol, theophylline
	HPLC	Theophylline, antibiotics
Theophylline	SC ^c	Diazepam, caffeine, metabolites in RF
	HPLC	Acetazolamide, cephalosporin antibiotics, endogenous xanthines, and theo metabolites in RF (minor elevations)
	GC	Phenobarbital (rare)
	IA	Caffeine, cross-reaction with metabolites in RF
Acetaminophen	SC	Salicylate, salicylamide, methyl salicylate (salicylate from these sources will inc at 10% of salicylate value in µg/mL), phenols, bilirubin, RF itself (for each inc mg/dL creatinine inc 30 µg/mL acetaminophen)
	GC	Phenacetin
	HPLC	Cephalosporins, sulfonamides
	IA	Phenacetin
Ethanol	SC ^c	Other alcohols, ketones (for oxidation methods), isopropanol (for enzymatic methods)
	GC	Few
Isopropanol	GC	Skin disinfectant containing isopropanol during venipuncture
Iron	SC	Deferoxamine causes 15% lowering of TIBC, lavendertop Vacutainer contains ethylenediaminetetraacetic acid (EDTA), which binds iron (lowers iron)
Lithium	SC	Green-top (lithium heparin) Vacutainer specimen tube
Digoxin	IA	Endogenous digoxin-like natriuretic substances (around 1 ng/mL in newborns, RF, pregnancy, liver disease, volume loading), cross-reaction with accumulated metabolites in RF (up to 2 ng/mL); oleander ingestion (glycosides may be detectable as digoxin)
Methemoglobin	SC	Sulfhemoglobin, methylene blue (15% unit increase after 2 mg/kg dose), hemoglobin regeneration is time-dependent (in Vacutainer around 10%/h and rapid following methylene blue)
Osmolality		Lavendertop (Ethylenediaminetetraacetic acid [EDTA]) Vacutainer tube (15 mOsm) graytop (NaF/K oxalate) tube (150 mOsm) bluetop (citrate) tube (10 mOsm)
	VP	Methanol, ethanol, acetone contribution to osm gap are underestimated

Source: Reprinted from Osterloh, J., *Emerg. Med. Clin. N. Am.*, 8, 693, 1990. With permission.

Abbreviations: SC, spectrochemical; RF, renal failure; GC, gas chromatography; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; IA, immunoassay; inc, increase; VP, vapor pressure; osm, osmolar.

^a All interferences listed do not occur in one assay but have been reported individually as interfering in one or another method variation. Interferences listed cause increase in measured drug, unless otherwise indicated.

^b More common in urine test.

^c Uncommon methodology.

ROLE OF THE POISON CONTROL CENTER

Poison Control Centers have had and continue to have a vital role in the provision of poison information since their inception in the 1950s. The regional poison centers (listed in Table 18.31) that are members of, or are certified by, the AAPCC serve several functions:

1. Provide expert information and consultation to the public and health professionals
2. Provide public education programs in poison prevention, counseling, and management

3. Provide regional professional education programs
4. Interact with prehospital care providers, healthcare facilities, and analytical toxicology laboratories to improve the management of the poisoned patient
5. Collect uniform data on poisonings and participate in nationwide sharing of data regarding poisonings

A single toll-free phone number (**1-800-222-1222**) has been established to allow individuals to reach a poison control center anywhere in the nation. Persons dialing the

number will be automatically linked to the closest poison center based on area code and exchange of the caller. The number is functional 24 h/day. Before this number was established, the country's poison control centers had more than 130 individual and separate phone numbers.

Most of the calls received are managed by poison information specialists who are either registered nurses, pharmacists, or other health-related professionals. Doctors of clinical pharmacy (Pharm.D.), clinical pharmacologists (PhD), and/or physicians who have had special training and/or board certifications are employed by most centers as managing directors to oversee and assist the information specialists WHO handle the exposure calls.

Table 18.26 provides data regarding the site of the caller and the site of human exposures reported to regional poison control centers in 1991 and 2010. Poison center call volumes are typically highest between 9:00 a.m. and 11:00 p.m. with a peak call volume between 4:00 and 11:00 p.m.

Tables 18.27 and 18.28 display symptom assessment and the subsequent site of management of the exposure cases. Importantly, more than two-third of cases are managed in a non-healthcare facility suggesting that significant health-care dollars are saved by using poison center services. Tables 18.29 and 18.30 depict medical outcome by age and by reason for exposures reported to AAPCC.

Under the leadership of AAPCC, many regional poison control centers have taken active roles in local and national lobbying efforts to enact legislation that could influence the number and severity of poisonings. The earliest efforts resulted in the Poison Prevention Packaging Act of 1970 requiring child-resistant closures on certain medications.

Toxicologists are also taking an active role in the evaluation of prescription medications being considered for a switch to over-the-counter status.

Poison centers have proven to be effective in reducing emergency room visits for suspected poisonings¹⁵ and thus have proven their value in healthcare cost containment. In addition, poison control centers reduce unnecessary health-care expenditures for poisonings and reduce the burden on our 911 systems and emergency transport services. It has been estimated that if there were no poison control center services, 70% of people would call 911 or an emergency medical service.¹⁶ For example, in 1992 the six poison control centers in New York State treated >91,000 poisoning exposures safely and effectively at home, by telephone management. These clients were spared unnecessary visits to hospital emergency departments.

Table 18.31 is an up-to-date list of poison control centers nationwide.

TABLE 18.26**Site of Caller and Site of Exposure to Human Poison Exposure Cases**

Location	Site of Caller (%)		Site of Exposure (%)	
	1991	2010	1991	2010
Residence	80.7	74.6	92.0	93.8
Workplace	1.5	1.2	2.5	1.6
Healthcare facility	15.6	17.5	0.6	0.3
School	0.7	0.5	0.9	1.2
Other	1.2	5.9	2.1	2.3
Unknown	0.4	0.4	1.8	0.9

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.27**Symptom Assessment at Time of Initial Call to Poison Center**

Symptom Assessment	No.	%
Asymptomatic	1,159,054	63.1
Symptomatic, related to exposure	518,912	28.2
Symptomatic, unrelated to exposure	31,915	1.7
Symptomatic, unknown if related	89,008	4.8
Unknown	39,050	2.1
Total	1,837,939	100.0

Source: Reprinted from Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992. With permission.

TABLE 18.28**Management Site of Human Exposure Cases**

Location	1991 (%)	2010 (%)
Non-healthcare facility	71.6	71.3
Healthcare facility	25.9	25.2
Other/unknown	2.5	3.5

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

TABLE 18.29**Medical Outcome of Human Poison Exposure Cases by Patient Age: 1991 and 2010**

Outcome	≤5 Years	6–12 Years	Teens ^a	Adults ^b	Unknown Child	Unknown Age	Total
No effect							
1991	21.1 (%)	1.2	0.8	3.4	— ^c	0.1	26.6
2010	12.7	1.1	1.0	4.3	0.04	0.05	19.2
Minor effect							
1991	8.0	1.6	1.7	10.8	—	0.3	22.1
2010	4.0	0.9	1.7	8.2	0.02	0.08	14.9
Moderate effect							
1991	0.3	0.1	0.2	1.6	—	0.03	2.2
2010	0.5	0.2	0.9	4.3	<0.01	0.02	5.9
Major effect							
1991	0.03	<0.01	0.02	0.3	—	<0.01	0.4
2010	0.03	<0.01	0.09	0.7	<0.01	<0.01	0.9
Death							
1991	<0.01	<0.01	<0.01	<0.04	—	0	0.04
2010	<0.01	<0.01	<0.01	<0.01	0.0	<0.01	<0.01
Unknown, no follow-up, nontoxic ^d							
1991	25.4	1.9	0.8	5.2	—	—	33.3
2010	9.8	0.9	0.4	2.3	0.03	0.03	13.5
Unknown, no follow-up, potentially toxic ^d							
1991	4.0	0.7	0.8	6.0	—	0.3	11.8
2010	23.0	2.8	2.3	14.6	0.1	0.4	43.2
Unrelated effect							
1991	0.9	0.2	0.1	1.3	—	0.02	2.5
2010	<.01	0.1	0.2	1.4	<0.01	0.02	1.7
Total 1991	59.7	5.7	4.4	28.6	—	0.8	
Total 2010	50.1	6.0	6.6	35.8	0.2	0.6	

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

^a 13–17 years for 1991; 13–19 years for 2010.

^b ≥18 years for 1991; ≥20 years for 2010.

^c Data not available.

^d Exposure assessed as nontoxic or potentially toxic.

TABLE 18.30
Medical Outcome by Reason for Exposure in Human Exposures: 1991 and 2010

Outcome	Unintentional/ Accidental	Intentional	Other	Adverse Reaction	Unknown	Total
No effect						
1991	24.8 (%)	1.8	— ^a	0.04	0.03	26.7
2010	16.7	2.3	0.1	0.06	0.05	19.2
Minor effect						
1991	17.8	3.7	—	0.7	0.1	22.3
2010	9.8	4.2	0.1	0.6	0.09	14.8
Moderate effect						
1991	1.3	0.9	—	0.1	0.03	2.3
2010	2.0	3.4	0.05	0.3	0.1	5.9
Major effect						
1991	0.1	0.2	—	<0.01	<.01	0.3
2010	0.1	0.6	<0.01	0.03	0.04	0.8
Death						
1991	<0.01	0.03	—	<0.01	<0.01	0.06
2010	<0.01	0.05	<0.01	<0.01	<0.01	0.09
Unknown, no follow-up, nontoxic ^b						
1991	32.7	0.9	—	0.3	0.1	34.0
2010	13.1	0.2	0.05	0.04	0.01	13.4
Unknown, no follow-up, potentially toxic ^b						
1991	8.6	2.8	—	0.3	0.1	11.8
2010	38.0	3.6	0.4	1.1	0.2	43.3
Unrelated effect						
1991	2.1	0.2	—	0.2	0.04	2.5
2010	1.7	0.3	0.06	0.4	0.06	2.5
Total 1991	87.4	10.5	—	1.7	0.4	
Total 2010	81.4	14.7	0.8	2.5	0.6	

Sources: Litovitz, T. et al., *Am. J. Emerg. Med.*, 10, 452, 1992; Bronstein, A.C. et al., *Clin. Toxicol.*, 49, 910, 2011.

^a Data not available.

^b Exposure assessed as nontoxic or potentially toxic.

TABLE 18.31
US Poison Control Centers

National Hotline: 1-800-222-1222 (Coordinates calls for the poison control centers)

Alabama

Alabama Poison Center

Regional Poison Control Center

Alaska

Served by Oregon Poison Control Center

Arizona

Arizona Poison and Drug Information Center

Banner Poison Control Center

Arkansas

Arkansas Poison and Drug Information Center

California

California Poison Control System—Fresno Division

California Poison Control System—Sacramento Division

California Poison Control System—San Diego Division

California Poison Control System—San Francisco Division

Colorado

Rocky Mountain Poison and Drug Center

Connecticut

Connecticut Poison Control Center

Delaware

Served by The Poison Control Center (Philadelphia)

District of Columbia

National Capital Poison Center

Florida

Florida Poison Information Center—Jacksonville

Florida Poison Information Center—Miami

Florida Poison Information Center—Tampa

Georgia

Georgia Poison Center

Hawaii

Served by: Rocky Mountain Poison and Drug Center (Colorado)

Idaho

Served by: Rocky Mountain Poison and Drug Center (Colorado)

Illinois

Illinois Poison Center

Indiana

Indiana Poison Center

Iowa

Iowa Statewide Poison Control Center

Kansas

University of Kansas Hospital Poison Control Center

Kentucky

Kentucky Regional Poison Center

Louisiana

Louisiana Poison Center

Maine

Northern New England Poison Center

TABLE 18.31 (continued)
US Poison Control Centers

Maryland

Maryland Poison Center

National Capital Poison Center

Massachusetts

Regional Center for Poison Control and Prevention

Michigan

Children's Hospital of Michigan Regional Poison Control Center

Minnesota

Hennepin Regional Poison Center

Mississippi

Mississippi Poison Control Center

Missouri

Missouri Poison Center

Montana

Served by Rocky Mountain Poison and Drug Center (Colorado)

Nebraska

Nebraska Regional Poison Center

Nevada

Served by Rocky Mountain Poison and Drug Center (Colorado)

New Hampshire

Served by: Northern New England Poison Center (Maine)

New Jersey

New Jersey Poison Information and Education System

New Mexico

New Mexico Poison and Drug Information Center

New York

New York City Poison Center

Upstate New York Poison Center

North Carolina

Carolinas Poison Center

North Dakota

Served by: Hennepin Regional Poison Center (Minnesota)

Ohio

Central Ohio Poison Center

Cincinnati Drug and Poison Information Center

Northern Ohio Poison Center

Oklahoma

Oklahoma Poison Control Center

Oregon

Oregon Poison Center

Pennsylvania

Pittsburgh Poison Center

The Poison Control Center (Philadelphia)

Rhode Island

Served by: Regional Center for Poison Control and Prevention (Boston)

South Carolina

Palmetto Poison Center

(continued)

TABLE 18.31 (continued)
US Poison Control Centers

South Dakota

Served by: Hennepin Regional Poison Center (Minnesota)

Tennessee

Tennessee Poison Center

Texas

North Texas Poison Center

Central Texas Poison Center

South Texas Poison Center

Southeast Texas Poison Center

West Texas Regional Poison Center

Texas Panhandle Poison Center

Utah

Utah Poison Control Center

Vermont

Served by: Northern New England Poison Center (Maine)

Virginia

Blue Ridge Poison Center

National Capital Poison Center (Washington, DC)

Virginia Poison Center

Washington

Washington Poison Center

West Virginia

West Virginia Poison Center

Wisconsin

Wisconsin Poison Center

Wyoming

Served by: Nebraska Regional Poison Center

U.S. Territories:

American Samoa

Served by: Nebraska Regional Poison Center

Federated States of Micronesia

Served by: Nebraska Regional Poison Center

Guam

Served by: Oregon Poison Center

Puerto Rico

Puerto Rico Poison Center

U.S. Virgin Islands

Served by: Florida/USVI Poison Information Center—Jacksonville

REFERENCES

1. Litovitz, T. et al., AAPCC Annual Report, *Am. J. Emerg. Med.*, 10, 452, 1992.
2. Goldfrank, L. et al., *Goldfrank's Toxicologic Emergencies*, 5th edn., Appleton & Lange, Norwalk, CT, 1994.
3. Haddad, L. and Winchester, J., *Clinical Management of Poisoning & Drug Overdose*, 2nd edn., WB Saunders, Philadelphia, PA, 1990.
4. Schlesselman, J. J., *Case Control Studies: Design, Conduct, Analysis*, Oxford University Press, New York, 1982.
5. Fletcher, R., *Clinical Epidemiology: The Essentials*, 2nd edn., Williams & Wilkins, Baltimore, MD, 1988.
6. Gamble, J. F. and Battigelli, M. C., Epidemiology, in *Patty's Industrial Hygiene and Toxicology*, 3rd revised edn., Vol. I, Clayton, G. D. and Clayton, F. E., Eds., John Wiley & Sons, New York, 1978, Chapter 5.
7. Tomenson, J. A., Epidemiology in relation to toxicology, in *General and Applied Toxicology*, 2nd edn., Ballantyne, B., Marrs, T. C., and Syversen, T., Eds., Grove's Dictionary, New York, 1999, Chapter 70.
8. Litovitz, T. and Manoguerra, A., Comparison of pediatric poisoning hazards: An analysis of 3.8 million exposure incidents. A report from the AAPCC, *Pediatrics*, 89, 999, 1992.
9. King, W. and Palmisano, P., Ingestion of prescription drugs by children: An epidemiologic study, *South Med. J.*, 82, 1468, 1989.
10. Woolf, A. and Lovejoy, F., Epidemiology of drug overdose in children, *Drug Saf.*, 9, 291, 1993.
11. Paulson, J. A., The epidemiology of injuries in adolescents, *Pediatr. Ann.*, 17, 84, 1988.
12. Klein-Schwartz, W. and Oderda, G., Poisoning in the elderly: Epidemiological, clinical and management considerations, *Drugs Aging*, 1, 67, 1991.
13. Woolf, A. et al., Serious poisonings among older adults: A study of hospitalization and mortality rates in Massachusetts 1983–1985, *Am. J. Public Health*, 80, 867, 1990.
14. Osterloh, J., Utility and reliability of emergency toxicologic testing, *Emerg. Med. Clin. N. Am.*, 8, 693, 1990.
15. National Committee for Injury Prevention and Control, *Injury Prevention: Meeting the Challenge*, Education Development Center, Inc., Oxford University Press, Oxford, U.K., 1989.
16. Annual Reports, New York State Regional Poison Control Centers.
17. Bronstein, A. C., Spyker, D. A., Cantilena, L. R., Green, J. L., Rumack, B. H., and Dart, R. C., 2010 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 28th Annual Report, *Clin. Toxicol.*, 49, 910, 2011.

19 Chemical Toxicology

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INTRODUCTION

This chapter presents information useful to the toxicologist working in chemical industries.

The section “General information” provides general information to categorize chemicals based on exposure and specific toxicities that raise particular concern. An overview of various OECD testing guidelines is presented as well as information related to the labeling and reporting of chemical safety information.

The section “Classification of chemicals based on toxicity” provides a comprehensive overview of various schemes for classifying chemicals based on acute lethality, repeated dose organ toxicity, reproductive and developmental effects, eye and skin irritation, skin and respiratory sensitization, mutagenicity, and carcinogenicity. Classifications can also be based on other endpoints such as environmental fate, chemical and physical hazards, etc., that are beyond the scope of this book. The reader is referred to the referenced literature for other classification schemes. Classification schemes are primarily utilized for hazard labeling that provides a general description of the hazards and relative potency of chemicals for individuals involved in their shipping, handling, use, and disposal.

Classification schemes are useful for ranking chemicals. However, caution should be exercised when using single classifications to communicate risk information or to make risk management decisions. For example, for a classification based solely on lethality, the median lethal dose is not the only determinant of toxicity. Consideration must also be given to the slope of the dose–response curve. Two substances having identical LD₅₀ values could vary significantly in the slope of their dose–response curves

and pose much different risks to hyperreactive individuals (see Figure 22.15). Moreover, the use of single classifications of this type can also relay a false sense of security because other determinants of toxicity are not addressed in a classification based on lethality alone. For example, a teratogenic substance could be classified as “slightly toxic” based solely on its LD₅₀. Classification schemes should only be used with a clear understanding of their inherent limitations.

Labeling and classification schemes are numerous and somewhat conflicting. There has been considerable effort to develop a system of globally harmonized schemes to unify the labeling and classification of chemicals. The Globally Harmonized System (GHS)¹ is becoming widely used as it is adopted by more national regulatory agencies worldwide. Previously used Risk (R) Phrases are being replaced by the new GHS Hazard (H) Statements. Despite some similarities, Risk Phrases do not directly convert to Hazard Statements. Many of the classification schemes presented in the section “Classification of chemicals based on toxicity” are no longer used or are being phased out by the GHS. They are included here for historical reasons to aid in the interpretation and understanding of older documents and reports that one may encounter. The GHS is being phased during a transition period but is being utilized at least in part by many countries as this book goes to press. The reader is advised to always verify the current requirements of regulatory agencies related to labeling and classification of chemicals.

GENERAL INFORMATION

See Tables 19.1 through 19.6.

TABLE 19.1
Criteria Defining “High-Exposure” Chemicals

- Production greater than 100,000 kg
- More than 1000 workers exposed
- More than 100 workers exposed by inhalation to greater than 10 mg/kg/day
- More than 100 workers exposed by inhalation to 1–10 mg/day for more than 100 days/year
- More than 250 workers exposed by routine dermal contact for more than 100 days/year
- Presence of the chemical in any consumer product in which the physical state of the chemical in the product and the manner of use would make exposure likely
- More than 70 mg/year of exposure via surface water
- More than 70 mg/year of exposure via air
- More than 70 mg/year of exposure via groundwater
- More than 10,000 kg/year release to environmental media
- More than 1000 kg/year total release to surface water after calculated estimates of treatment

Source: U.S. Environmental Protection Agency, Reported in *Pesticide and Toxic Chemical News*, 19, 34, October 1988.

TABLE 19.2
Selected OECD Guidelines for Testing of Chemicals

Mammalian		Genetox	
#401	Acute oral toxicity, LD ₅₀	#471	Reverse mutation assay/ <i>Salmonella</i> (Ames test)
#402	Acute dermal toxicity	#473	<i>In vitro</i> mammalian chromosome aberration test
#403	Acute inhalation toxicity	#474	Micronucleus test
#404	Acute dermal irritation/corrosion	#475	<i>In vivo</i> bone marrow mammalian chromosome aberration test
#405	Acute eye irritation	#476	<i>In vitro</i> mammalian cell gene mutation test (mouse lymphoma)
#406	Contact sensitization	#477	Sex-linked recessive lethal test (<i>Drosophila</i>)
#407	Repeated dose 28 day oral toxicity (rodent)	#478	Rodent dominant lethal test (mouse)
#408	Repeated dose 90 day oral toxicity (rodent)	#479	<i>In vitro</i> sister chromatid exchange (SCE) assay
#409	Repeated dose 90 day oral toxicity (nonrodent)	#480	Gene mutation assay/ <i>Saccharomyces</i>
#410	Repeated dose 28 day dermal toxicity	#481	Mitotic recombination assay/ <i>Saccharomyces</i>
#411	Repeated dose 90 day dermal toxicity	#482	<i>In vivo</i> unscheduled DNA synthesis (UDS)
#412	Repeated dose 28 day inhalation toxicity	#483	Mammalian spermatogonial chromosome aberration assay
#413	Repeated dose 90 day inhalation toxicity	#484	Mouse spot test
#414	Developmental toxicity study	#485	Mouse heritable translocation assay
#415	One generation reproduction		
#416	Two generation reproduction		Ecotox/Aquatic
#417	Metabolism and pharmacokinetics	#106	Absorption/desorption
#418	Acute exposure delayed neurotoxicity of organophosphorus substances	#201	Algal growth inhibition
#419	Repeated exposure delayed neurotoxicity of organophosphorus substances	#202	Acute toxicology/ <i>Daphnia</i>
#420	Acute oral toxicity, fixed dose procedure	#203	Acute toxicity/fish
#421	Reproductive/developmental toxicity screen	#204	14 day prolonged toxicity/fish
#422	Combined repeated dose 28 day oral toxicity/developmental toxicity screen	#205	Avian dietary toxicity
#423	Acute oral toxicity, acute toxic class	#206	Avian reproduction
#424	Neurotoxicity screening battery	#207	Acute toxicity/earthworm
#425	Acute oral toxicity, up and down method	#208	Terrestrial plant growth test
#429	Skin sensitization (local lymph node assay)	#209	Activated sludge respiration inhibition
#436	Acute inhalation toxicity–acute toxic class method	#210	Fish early life stage toxicity
#451	Carcinogenicity	#211	21 day <i>Daphnia</i> reproduction
#452	Chronic toxicity	#215	Fish juvenile growth test
#453	Combined chronic toxicity/carcinogenicity	#222	Earthworm reproduction test
		#223	Avian acute oral toxicity
		#229	Fish short-term reproduction assay
		#230	21 day fish assay
		#301	Ready biodegradability
		#302	Inherent biodegradability
		#305	Bioaccumulation/fish

TABLE 19.3
EPA Categories of Toxicological Concerns^a

Category	Concern
Acid chlorides	<i>Environmental toxicity.</i> ^b Concern is greater if the log octanol/water partition coefficient ($\log K_{ow}$) <8 or if molecular weight (mol. wt.) <1000
Acrylamides	<i>Human health and environmental toxicity.</i> The acrylamides of greatest concern are those with a labile substituent, for example, methylol acrylamides, that may release acrylamide <i>per se</i> under metabolic conditions Members of this class are considered potential carcinogens, heritable mutagens, developmental and reproductive toxicants, and are potential neurotoxins Structures with an acrylamide equivalent weight ≥ 5000 are presumed <i>not</i> to pose a hazard under any condition
Acrylates and methacrylates	<i>Environmental toxicity.</i> Particularly if the log of the octanol/water partition coefficient ($\log P$) <5. Concerns typically confined to species with mol. wt. <1000 <i>Human health.</i> Some individual compounds are irritants and sensitizers
Aldehydes	<i>Environmental toxicity.</i> Generally mol. wt. <1000, $\log K_{ow} < 8$
Aliphatic amines	<i>Environmental toxicity.</i> Generally mol. wt. <1000
Alkoxy silanes	<i>Human health and environmental toxicity.</i> There is a concern for lung toxicity of such substances if inhaled
Aluminum compounds	<i>Environmental toxicity.</i> Soluble forms of aluminum especially with solubility >1 ppb
Anhydrides, carboxylic acid	<i>Human health.</i> Potential for pulmonary sensitization; also developmental or reproductive toxicity. Generally if mol. wt. <1000
Anilines	<i>Environmental toxicity.</i> Generally if $\log K_{ow} < 8$ and mol. wt. <1000
Azides	<i>Environmental toxicity.</i> Generally mol. wt. <1000, $\log K_{ow} < \sim 8$
Benzotriazoles	<i>Environmental toxicity.</i> Generally mol. wt. <1000, $\log K_{ow} < 8$ are expected to manifest toxicity
Benzotriazole-hindered phenols	<i>Human health and environmental toxicity.</i> Liver, kidney, hematological and immune system effects
Boron compounds	<i>Human health and environmental toxicity.</i> Male and female reproductive toxicity, hematotoxicity, neurotoxicity
Cobalt and compounds	<i>Environmental toxicity.</i> Generally if $\log K_{ow} < 8.0$, mol. wt. is <1000 and water solubility >1 ppb
Dianilines	<i>Human health and environmental toxicity.</i> Potential carcinogens and mutagens. Also potential retinotoxic agents, reproductive and systemic toxicants
Diazoniums (aromatic only)	<i>Environmental toxicity.</i> Those with mol. wt. <1000 are of concern
Dichlorobenzidine-based pigments	<i>Human health and environmental toxicity.</i> Concern for mutagenicity/carcinogenicity
Diisocyanates (two or more isocyanate groups)	<i>Human health.</i> Potential dermal and pulmonary sensitization and other lung effects. Some may be carcinogenic. Structures with an isocyanate equivalent weight of ≥ 5000 are presumed <i>not</i> to pose a hazard under any conditions
Dithiocarbamates	<i>Environmental toxicity.</i> Generally mol. wt. <1000, $\log K_{ow} < 19$
Dyes: acid and amphoteric	<i>Environmental toxicity.</i> Particularly if the substance is water-soluble and mol. wt. is around 1000 or less
Dyes: cationic	<i>Environmental toxicity.</i> Any dye bearing one or more net positive charges. No mol. wt. threshold
Dyes: aminobenzothiazole (AZO)	<i>Human health and environmental toxicity.</i> There are mutagenicity/carcinogenicity concerns. There is also potential for liver, thyroid, and neurotoxicity. Ecotoxicity concerns generally relate to chronic toxicity
Epoxides	<i>Human health and environmental toxicity.</i> Concerns for cancer and reproductive effects. Structures with epoxy equivalent weights ≥ 1000 are presumed <i>not</i> to pose a hazard under any conditions
Esters	<i>Environmental toxicity.</i> Compounds with mol. wt. >1000 are not of concern
Ethylene glycol ethers	<i>Human health.</i> Irritation of skin, eyes, and mucous membranes; hemolysis, bone marrow damage, and leukopenia of both lymphocytes and granulocytes; direct and indirect kidney damage; liver damage, immunotoxicity, and central nervous system depression. Also developmental and reproductive toxicants
Hindered amines	<i>Human health.</i> May be toxic to the immune system, liver, blood, the male reproductive system, and the gastrointestinal tract
Hydrazines and related compounds	<i>Human health and environmental toxicity.</i> Concerns for carcinogenicity and chronic effects to liver, kidney, and blood
Imides	<i>Environmental toxicity.</i> Compounds with mol. wt. <1000, $\log K_{ow} \leq 8$ are of greater concern
Lanthanides or rare earth metals	<i>Environmental toxicity.</i> mol. wt. <1000

(continued)

TABLE 19.3 (continued)
EPA Categories of Toxicological Concerns

Category	Concern
β -Naphthylamines (monosulfonated)	<i>Human health.</i> Potential mutagens and carcinogens. Concern is restricted to those compounds where not more than two sulfonate or sulfatoethylsulfone groups are on the ring <i>distal</i> to the β -amino group
Neutral organics	<i>Environmental toxicity.</i> The molecular weights of neutral organics of concern are generally <1000 and the octanol/water partition coefficients ($\log P$) are <8
Nickel compounds	<i>Human health and environmental toxicity.</i> Concern for genotoxicity/carcinogenicity, fetotoxicity, and dermatotoxicity
Nitriles, allylic/vinyl	<i>Environmental toxicity.</i> Mol. wt. <1000, $\log K_{ow} \leq 8$
Organotin	<i>Human health and environmental toxicity.</i> Eye and skin irritants, systemic effects (primarily neurotoxicity), immunotoxicity. Some organotin are probable human carcinogens
Peroxides	<i>Human health and environmental toxicity.</i> Compounds assessed on a case-by-case basis
Persistent, bioaccumulative, and toxic (PBT) chemicals (e.g., DDT)	<i>Human health, environmental toxicity, and fate.</i> PBT chemical substances are chemicals that partition to water, sediment, or soil and are not removed at rates adequate to prevent their accumulation in aquatic or terrestrial species, with the potential to pose a risk via food chain toxicity. Concern for chemicals with persistence (transformation half-life) >2 months, bioaccumulation ≥ 1000 ($\log K_{ow} = 4.2$) and mol. wt. <1000
Phenolphthaleins	<i>Human health.</i> Concern for carcinogenicity
Phenols	<i>Environmental toxicity.</i> Compounds of greater concern have mol. wt. <1000
Phosphates, inorganic	<i>Environmental toxicity.</i> High concern for eutrophication
Phosphinate esters	<i>Environmental toxicity.</i> Generally if $\log K_{ow} \leq 8.0$, mol. wt. is ≤ 1000
Polyanionic polymers (and monomers)	<i>Environmental toxicity.</i> Compounds must be water-soluble or water self-dispersing to be in this category. Mol. wt. can be >1000, $\log K_{ow} < \sim 10$
Polycationic polymers	<i>Environmental toxicity.</i> The polymers must be water-soluble or water-dispersible and the molecular weights are generally >300
Polynitroaromatics	<i>Environmental toxicity.</i> Concern is for compounds with mol. wt. <1000
Respirable, poorly soluble particulates	<i>Human health.</i> Particles $\leq 10 \mu\text{m}$. Effects on the lung ranging from inflammation to fibrosis and potentially cancer
Rosin	<i>Environmental toxicity.</i> Category includes rosin, abietic acid, abietinic acid and their salts, and polymeric forms with mol. wt. <1000
Stilbene, derivatives of 4,4-bis(triazin-2-ylamino)-	<i>Human health.</i> Evaluated on a case-by-case basis
Thiols/mercaptans	<i>Environmental toxicity.</i> Mol. wt. < 1000, $\log K_{ow} < \sim 9$
Triaryl methane pigments/dyes with insoluble groups	<i>Human health and environmental toxicity.</i> Developmental/reproductive toxicity and carcinogenicity concerns
Substituted triazines	<i>Environmental toxicity.</i> If $\log K_{ow} \leq 8.0$, mol. wt. is <1000
Surfactants: anionic	<i>Environmental toxicity.</i> No mol. wt. boundary
Surfactants: cationic (quaternary ammonium)	<i>Environmental toxicity.</i> Little ecotoxicity is expected when the carbon chain length exceeds 22 carbons
Surfactants: nonionic	<i>Environmental toxicity.</i> Acute aquatic toxicity increases with the hydrophobic chain length when the number of ethoxy groups or the hydrophilic component is held constant. Aquatic toxicity is decreased with increasing number of ethoxylate groups when the number of carbons in the hydrophobe is constant
Vinyl esters	<i>Human health and environmental toxicity.</i> Concern for carcinogenicity, neurotoxicity, and reproductive toxicity
Vinyl sulfones	<i>Human health and environmental toxicity.</i> Concern for mutagenicity and carcinogenicity
Soluble complexes of zinc	<i>Environmental toxicity</i>
Zirconium compounds	<i>Environmental toxicity.</i> Concern for mol. wt. <1000

Source: U.S. Environmental Protection Agency, *TSCA New Chemicals Program (NCP) Chemical Categories*, Office of Pollution Prevention and Toxics, August 2010.

^a This table is an overview of toxicological concerns for various chemical classes. The reader is referred to the reference which is available on the EPA website for detailed information on hazard concerns and physical/chemical boundaries for these concerns.

^b Environmental toxicity usually indicates toxicity to algae, daphnids, and/or fish although concerns for other environmental effects could exist.

TABLE 19.4

Risk (R) Phrases Used in the European Community (EU)

R1	Explosive when dry	R50	Very toxic to aquatic organisms
R2	Risk of explosion by shock, friction, fire, or other sources of ignition	R51	Toxic to aquatic organisms
R3	Extreme risk of explosion by shock, friction, fire, or other sources of ignition	R52	Harmful to aquatic organisms
R4	Forms very sensitive explosive metallic compounds	R53	May cause long-term adverse effects in the aquatic environment
R5	Heating may cause an explosion	R54	Toxic to flora
R6	Explosive with or without contact with air	R55	Toxic to fauna
R7	May cause fire	R56	Toxic to soil organisms
R8	Contact with combustible material may cause fire	R57	Toxic to bees
R9	Explosive when mixed with combustible material	R58	May cause long-term adverse effects to the environment
R10	Flammable	R59	Dangerous for the ozone layer
R11	Highly flammable	R60	May impair fertility
R12	Extremely flammable	R61	May cause harm to the unborn child
R14	Reacts violently with water	R62	Possible risk of impaired fertility
R15	Contact with water liberates extremely flammable gases	R63	Possible risk of harm to the unborn child
R16	Explosive when mixed with oxidizing substances	R64	May cause harm to breast-fed babies
R17	Spontaneously flammable in air	R68	Possible risk of irreversible effects
R18	In use may form flammable/explosive vapor–air mixture	Combination of Particular Risks	
R19	May form explosive peroxides	R14/15	Reacts violently with water, liberating extremely flammable gases
R20	Harmful by inhalation	R15/29	Contact with water liberates toxic, extremely flammable gas
R21	Harmful in contact with skin	R20/21	Harmful by inhalation and in contact with skin
R22	Harmful if swallowed	R20/21/22	Harmful by inhalation, in contact with skin and if swallowed
R23	Toxic by inhalation	R20/22	Harmful by inhalation and if swallowed
R24	Toxic in contact with skin	R21/22	Harmful in contact with skin and if swallowed
R25	Toxic if swallowed	R23/24	Toxic by inhalation and in contact with skin
R26	Very toxic by inhalation	R23/24/25	Toxic by inhalation, in contact with skin, and if swallowed
R27	Very toxic in contact with skin	R23/25	Toxic by inhalation and if swallowed
R28	Very toxic if swallowed	R24/25	Toxic in contact with skin and if swallowed
R29	Contact with water liberates toxic gas	R26/27	Very toxic by inhalation and in contact with skin
R30	Can become highly flammable in use	R26/27/28	Very toxic by inhalation, in contact with skin and if swallowed
R31	Contact with acids liberates toxic gas	R26/28	Very toxic by inhalation and if swallowed
R32	Contact with acids liberates very toxic gas	R27/28	Very toxic in contact with skin and if swallowed
R33	Danger of cumulative effects	R36/37	Irritating to eyes, respiratory system
R34	Causes burns	R36/37/38	Irritating to eyes, respiratory system, and skin
R35	Causes severe burns	R36/38	Irritating to eyes and skin
R36	Irritating to the eyes	R37/38	Irritating to respiratory system and skin
R37	Irritating to the respiratory system	R39/23	Toxic: danger of very serious irreversible effects through inhalation
R38	Irritating to the skin	R39/23/24	Toxic: danger of very serious irreversible effects through inhalation and in contact with skin
R39	Danger of very serious irreversible effects	R39/23/24/25	Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed
R40	Limited evidence of a carcinogenic effect	R39/23/25	Toxic: danger of very serious irreversible effects through inhalation and if swallowed
R41	Risk of serious damage to the eyes	R39/24	Toxic: danger of very serious irreversible effects in contact with skin
R42	May cause sensitization by inhalation	R39/24/25	Toxic: danger of very serious irreversible effects in contact with skin and if swallowed
R43	May cause sensitization by skin contact		
R44	Risk explosion if heated under confinement		
R45	May cause cancer		
R46	May cause heritable genetic damage		
R48	Danger of serious damage to health by prolonged exposure		
R49	May cause cancer by inhalation		

(continued)

TABLE 19.4 (continued)

Risk (R) Phrases Used in the European Community (EU)

R39/25	Toxic: danger of very serious irreversible effects if swallowed	R48/20/21	Harmful: danger of serious damage to health by prolonged exposure through inhalation and in contact with skin
R39/26	Very toxic: danger of very serious irreversible effects through inhalation	R48/20/21/22	Harmful: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin, and if swallowed
R39/26/27	Very toxic: danger of very serious irreversible effects through inhalation and in contact with skin	R48/20/22	Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
R39/26/27/28	Very toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed	R48/21	Harmful: danger of serious damage to health by prolonged exposure in contact with skin
R39/26/28	Very toxic: danger of very serious irreversible effects through inhalation and if swallowed	R48/21/22	Harmful: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed
R39/27	Very toxic: danger of very serious irreversible effects in contact with skin	R48/22	Harmful: danger of serious damage to health by prolonged exposure if swallowed
R39/27/28	Very toxic: danger of very serious irreversible effects in contact with skin and if swallowed	R48/23	Toxic: danger of serious damage to health by prolonged exposure through inhalation
R39/28	Very toxic: danger of very serious irreversible effects if swallowed	R48/23/24	Toxic: danger of serious damage to health by prolonged exposure through inhalation and in contact with skin
R68/20	Harmful: possible risk of irreversible effects through inhalation	R48/23/24/25	Toxic: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin, and if swallowed
R68/20/21	Harmful: possible risk of irreversible effects through inhalation and in contact with skin	R48/23/25	Toxic: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
R68/20/21/22	Harmful: possible risk of irreversible effects through inhalation, in contact with skin, and if swallowed	R48/24	Toxic: danger of serious damage to health by prolonged exposure in contact with skin
R68/20/22	Harmful: possible risk of irreversible effects through inhalation and if swallowed	R48/24/25	Toxic: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed
R68/22	Harmful: possible risk of irreversible effects if swallowed	R48/25	Toxic: danger of serious damage to health by prolonged exposure if swallowed
R68/21	Harmful: possible risk of irreversible effects in contact with skin	R50/53	Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R68/21/22	Harmful: possible risk of irreversible effects in contact with skin and if swallowed	R51/53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R42/43	May cause sensitization by inhalation and skin contact	R52/53	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R48/20	Harmful: danger of serious damage to health by prolonged exposure through inhalation		

TABLE 19.5
GHS Hazard (H) Statements

Physical Hazards			
H200	Unstable explosive	H311	Toxic in contact with skin
H201	Explosive; mass explosion hazard	H312	Harmful in contact with skin
H202	Explosive; severe projection hazard	H313	May be harmful in contact with skin
H203	Explosive; fire, blast, or projection hazard	H314	Causes severe burns and eye damage
H204	Fire or projection hazard	H315	Causes skin irritation
H205	May mass explode in fire	H316	Causes mild skin irritation
H220	Extremely flammable gas	H317	May cause an allergic skin reaction
H221	Flammable gas	H318	Causes serious eye damage
H222	Extremely flammable aerosol	H319	Causes serious eye irritation
H223	Flammable aerosol	H320	Causes eye irritation
H224	Extremely flammable liquid and vapor	H330	Fatal if inhaled
H225	Highly flammable liquid and vapor	H331	Toxic if inhaled
H226	Flammable liquid and vapor	H332	Harmful if inhaled
H227	Combustible liquid	H333	May be harmful if inhaled
H228	Flammable solid	H334	May cause allergy or asthma symptoms or breathing difficulties if inhaled
H240	Heating may cause an explosion	H335	May cause respiratory irritation
H241	Heating may cause a fire or explosion	H336	May cause drowsiness or dizziness
H242	Heating may cause a fire	H340	May cause genetic defects
H250	Catches fire spontaneously if exposed to air	H341	Suspected of causing genetic defects
H251	Self-heating; may catch fire	H350	May cause cancer
H252	Self-heating in large quantities; may catch fire	H351	Suspected of causing cancer
H260	In contact with water releases flammable gases which may ignite spontaneously	H360	May damage fertility or the unborn child
H261	In contact with water releases flammable gas	H361	Suspected of damaging fertility or the unborn child
H270	May cause or intensify fire; oxidizer	H362	May cause harm to breast-fed children
H271	May cause fire or explosion; strong oxidizer	H370	Causes damage to organs
H272	May intensify fire; oxidizer	H371	May cause damage to organs
H280	Contains gas under pressure; may explode	H372	Causes damage to organs through prolonged or repeated exposure
H281	Contains refrigerated gas; may cause cryogenic burns or injury	H373	May cause damage to organs through prolonged or repeated exposure
H290	May be corrosive to metals	Environmental Hazards	
Health Hazards		H400	Very toxic to aquatic life
H300	Fatal if swallowed	H401	Toxic to aquatic life
H301	Toxic if swallowed	H402	Harmful to aquatic life
H302	Harmful if swallowed	H410	Very toxic to aquatic life and long-lasting effects
H303	May be harmful if swallowed	H411	Toxic to aquatic life with long-lasting effects
H304	May be fatal if swallowed and enters airways	H412	Harmful to aquatic life with long-lasting effects
H305	May be harmful if swallowed and enters airways	H413	May cause long-lasting harmful effects to aquatic life
H310	Fatal in contact with skin	H420	Harms public health and the environment by destroying ozone in the upper atmosphere

TABLE 19.6
Information Disclosed on a Safety Data Sheet (SDS)^a

Section 1	Chemical Product and Company Identification Product Name Generic Names/Synonyms Product Use Manufacturer's Name and Address Name and Phone Number of the Person/Group Who Prepared the MSDS Date MSDS was Prepared Emergency Phone Number	Section 9	Physical and Chemical Properties Appearance Boiling Point Physical State Melting Point Odor Vapor Pressure Specific Gravity Vapor Density Solubility Evaporation Rate pH % Volatiles
Section 2	Hazards Identification Potential Human Health Hazards To Skin (irritancy, sensitization) To Eyes (irritancy) via Inhalation (acute effects) via Ingestion (acute effects) Delayed Effects (chronic effects) Carcinogenicity, reproductive and developmental effects, mutagenicity, other	Section 10	Stability and Reactivity Stability Conditions Incompatibilities Hazardous Decomposition Products Hazardous Polymerization
Section 3	Composition/Information on Ingredients Ingredient Name(s) CAS Number(s) Percent by Weight	Section 11	Toxicity Information Acute Effects (LD ₅₀ , LC ₅₀) Subchronic and Chronic Effects Irritancy Sensitization Neurotoxicity Reprotoxicity Developmental Toxicity Mutagenicity
Section 4	First Aid Measures Specific First Aid Measures for Various Routes of Exposure Notes to Physician Including Antidotes and Medical Conditions Affected by the Product	Section 12	Ecological Information Aquatic Toxicity Terrestrial Toxicity Bioaccumulation Potential Biodegradability Microbial Toxicity
Section 5	Fire Fighting Measures Flammable Properties (flashpoint, autoignition temperature, etc.) Extinguishing Media Hazardous Combustion Products Explosion Hazards Firefighting Precautions and Instructions	Section 13	Disposal Information
Section 6	Accidental Release Measures Procedures to be Followed in Case of Spill or Other Release	Section 14	Shipping Information D.O.T. Hazard Class D.O.T. I.D. Number
Section 7	Handling and Storage Normal Handling Procedures Storage Recommendations	Section 15	Regulatory Information TSCA Inventory Status Other Federal, State, Local Foreign Regulatory Information
Section 8	Exposure Controls/Personal Protection Engineering Controls Personal Protective Equipment Exposure Guidelines (TLV, PEL, other)	Section 16	Information not covered in the other 15 sections

^a Formerly known as Material Safety Data Sheet (MSDS)

CLASSIFICATION OF CHEMICALS BASED ON TOXICITY

See Tables 19.7 through 19.13 and Figures 19.1 through 19.5.

TABLE 19.7
Combined Tabulation of Toxicity Classes

Toxicity Rating	Commonly Used Term	Various Routes of Administration			
		LD ₅₀ Single Oral Dose Rats	Inhalation 4 h Vapor Exposure Mortality 2/6–4/6 Rats	LD ₅₀ Skin Rabbits	Probable Lethal Dose for Man
1	Extremely toxic	≤1 mg/kg	<10 ppm	≤5 mg/kg	A taste, 1 grain
2	Highly toxic	1–50 mg	10–100 ppm	5–43 mg/kg	1 teaspoon, 4 cc
3	Moderately toxic	50–500 mg	100–1,000 ppm	44–340 mg/kg	1 ounce, 30 g
4	Slightly toxic	0.5–5 g	1,000–10,000 ppm	0.35–2.81 g/kg	1 cup, 250 g
5	Practically nontoxic	5–15 g	10,000–100,000 ppm	2.82–22.59 g/kg	1 quart, 1000 g
6	Relatively harmless	>15 g	>100,000 ppm	>22.6 g/kg	>1 quart

Source: Hodge, H.C. and Sterner, J.H., *Am. Ind. Hygiene Assoc. Q.*, 10, 4, 1949. With permission.

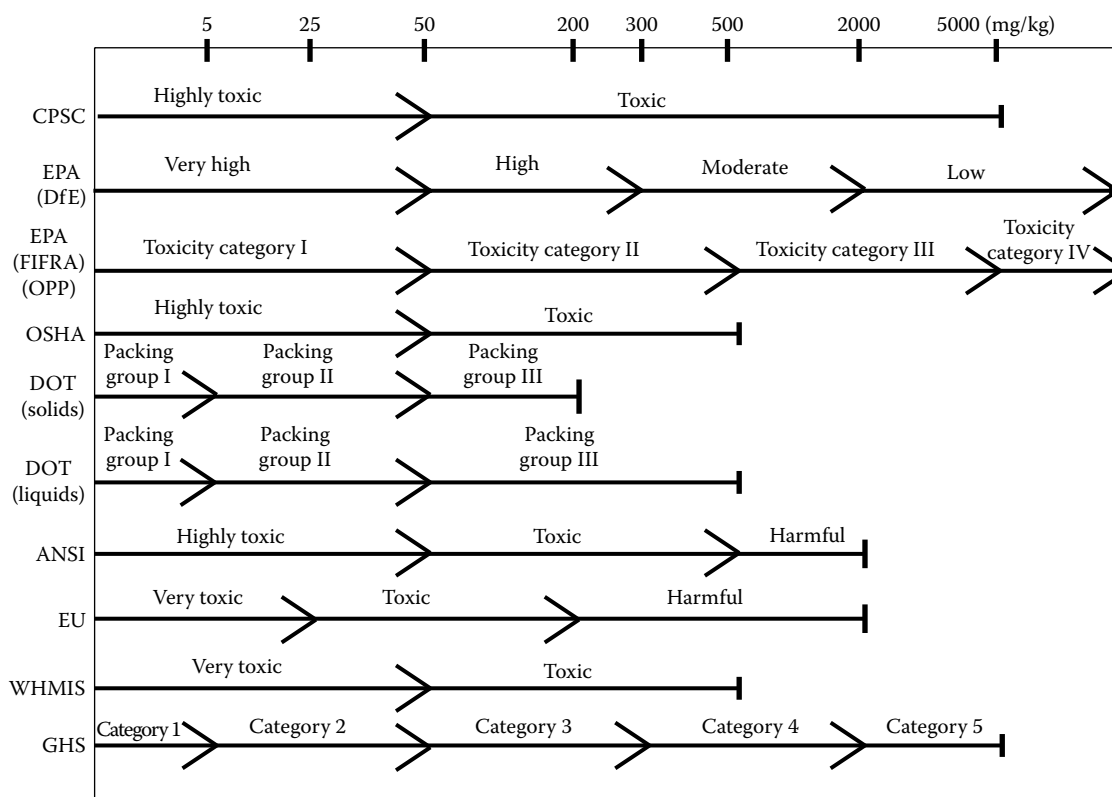


FIGURE 19.1 Classification based on rat acute oral LD₅₀. GHS Category 5 can also include chemicals for which assignment to more hazardous class is not warranted but there are indications of significant effects in humans and/or some mortality and/or significant clinical signs of toxicity within the Category 4 dose range. Refer to United Nations (2011) for specifics. CPSC, Consumer Product Safety Commission; EPA (DfE), U.S. Environmental Protection Agency (Design for the Environment Program); EPA (FIFRA), U.S. Environmental Protection Agency (Federal Insecticide, Fungicide and Rodenticide Act); EPA (OPP), U.S. Environmental Protection Agency (Office of Pesticide Programs); OSHA, U.S. Occupational Safety and Health Administration; DOT, U.S. Department of Transportation; ANSI, American National Standards Institute; EU, European Union; WHMIS, Workplace Hazardous Materials Information System (Canada); GHS, United Nations Globally Harmonized System. Use the following example of the DOT (solids) classification as an aid for interpreting the values of this figure: Packing Group I (≤5 mg/kg); Packing Group II (>5 to ≤50 mg/kg); Packing Group III (>50 to ≤200 mg/kg). (Adapted from Schurjer, M.G. and McConnell, F., *Eastman Chemicals*, Kingsport, TN, 1989.)

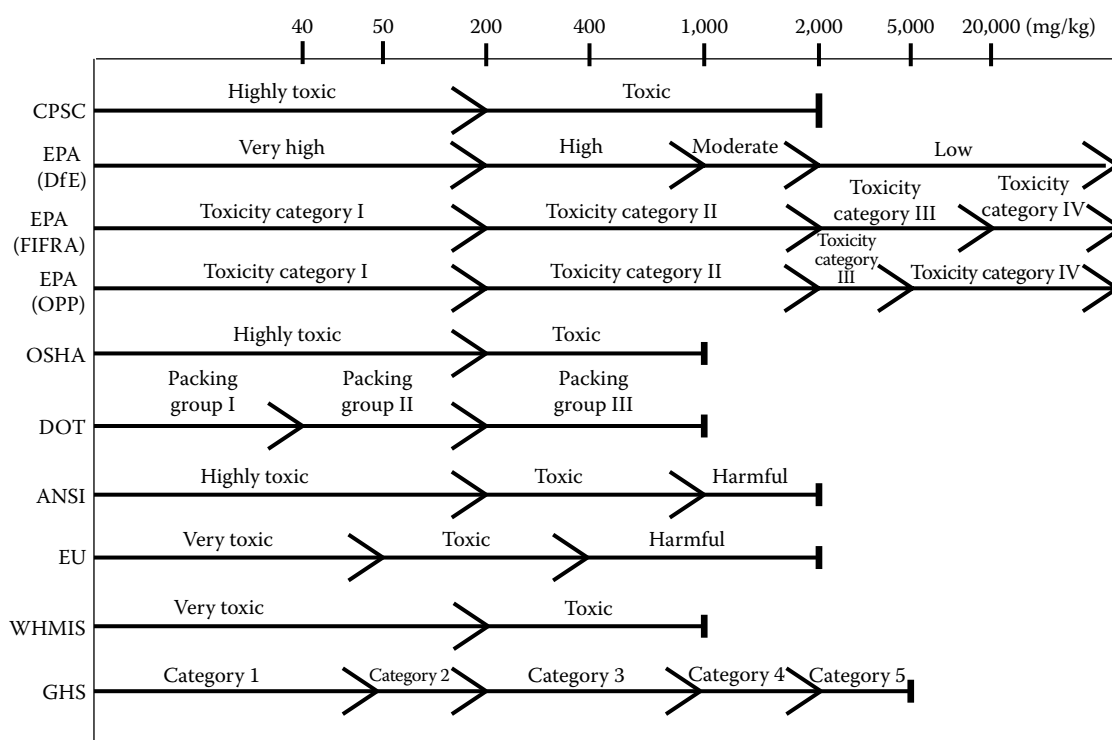


FIGURE 19.2 Classification based on rabbit or rat acute dermal LD₅₀. CPSC, Consumer Product Safety Commission; EPA (DfE), U.S. Environmental Protection Agency (Design for the Environment Program); EPA (FIFRA), U.S. Environmental Protection Agency (Federal Insecticide, Fungicide and Rodenticide Act); EPA (OPP), U.S. Environmental Protection Agency (Office of Pesticide Programs); OSHA, U.S. Occupational Safety and Health Administration; DOT, U.S. Department of Transportation; ANSI, American National Standards Institute; EU, European Union; WHMIS, Workplace Hazardous Materials Information System (Canada); GHS, United Nations Globally Harmonized System. Refer to the legend for Figure 19.1 for an aid to interpreting the values of this figure. (Adapted from Schurmer, M.G. and McConnell, F., *Eastman Chemicals*, Kingsport, TN, 1989.)

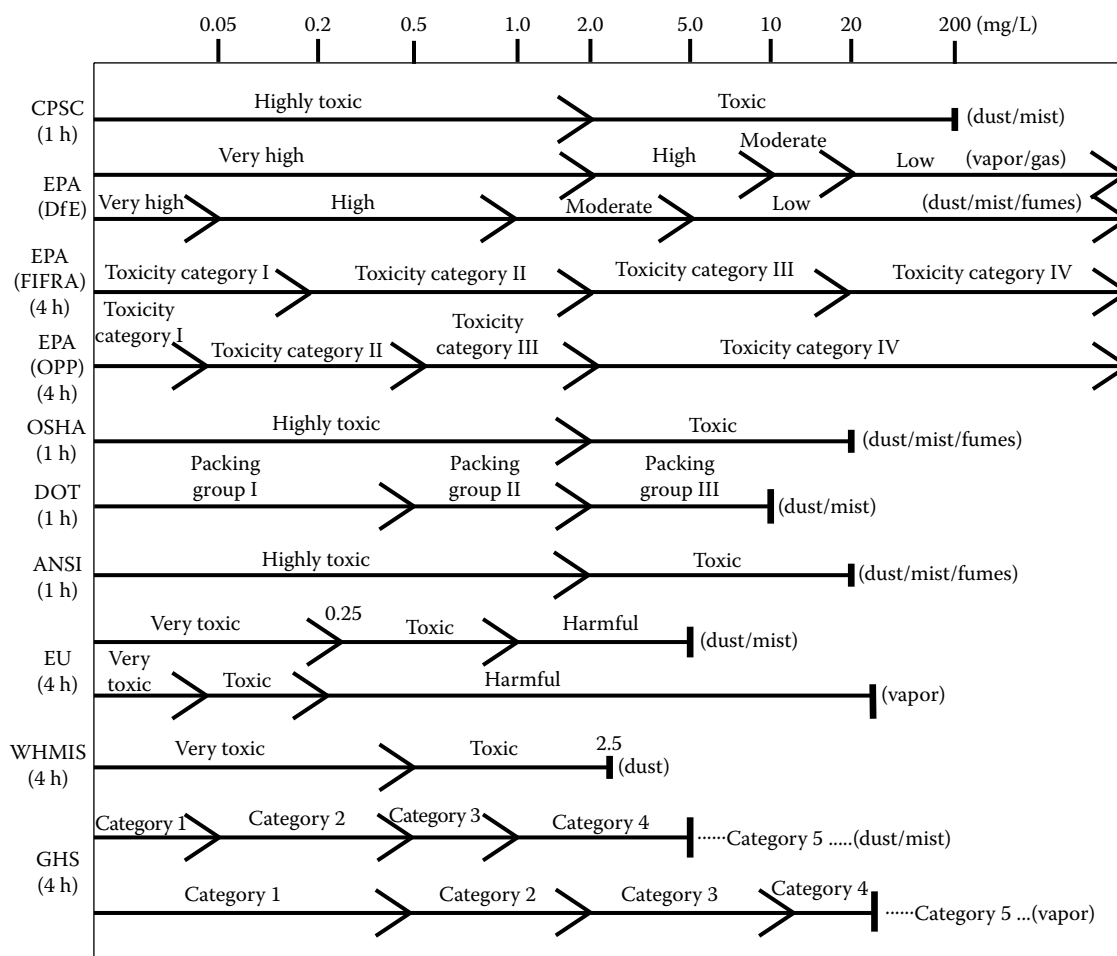


FIGURE 19.3 Classification based on rat acute inhalation LC_{50} (mg/L). CPSC, Consumer Product Safety Commission; EPA (DfE), U.S. Environmental Protection Agency (Design for the Environment Program); EPA (FIFRA), U.S. Environmental Protection Agency (Federal Insecticide, Fungicide and Rodenticide Act); EPA (OPP), U.S. Environmental Protection Agency (Office of Pesticide Programs); OSHA, U.S. Occupational Safety and Health Administration; DOT, U.S. Department of Transportation; ANSI, American National Standards Institute; EU, European Union; WHMIS, Workplace Hazardous Materials Information System (Canada); GHS, United Nations Globally Harmonized System. Refer to the legend for Figure 19.1 for an aid to interpreting the values of this figure. GHS Category 5 can include chemicals with LC_{50} s in the range equivalent to an oral/dermal LD_{50} between 2000 and 5000 mg/kg. Refer to United Nations (2011) for specifics. (Adapted from Schurger, M.G. and McConnell, F., *Eastman Chemicals*, Kingsport, TN, 1989.)

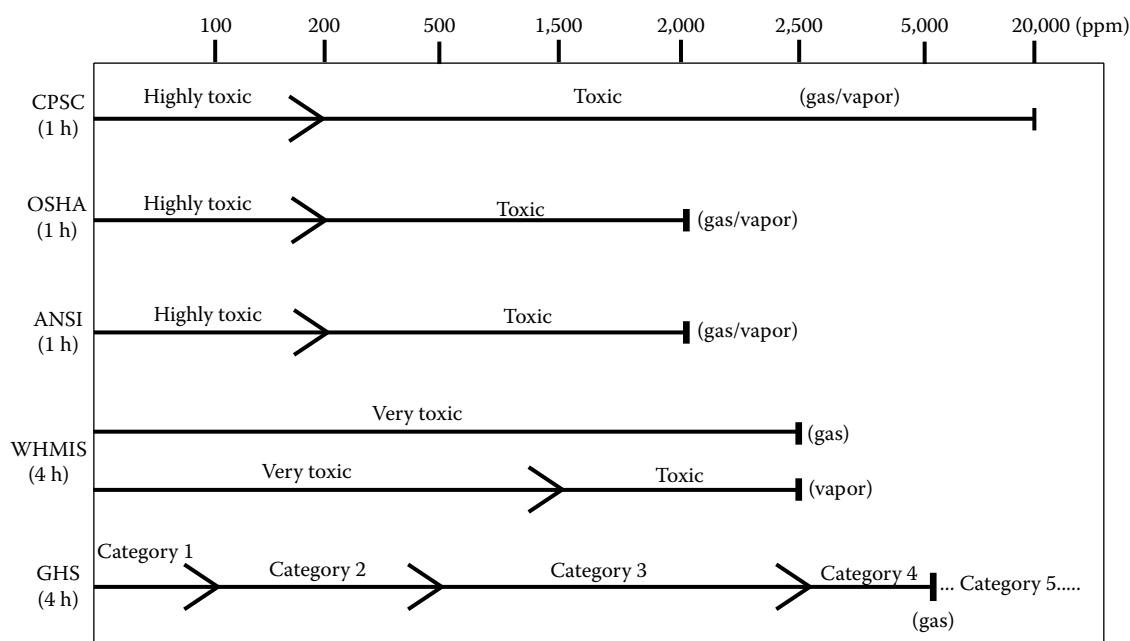


FIGURE 19.4 Classification based on rat acute inhalation LC_{50} (ppm). CPSC, Consumer Product Safety Commission; OSHA, U.S. Occupational Safety and Health Administration; ANSI, American National Standards Institute; WHMIS, Workplace Hazardous Materials Information System (Canada); GHS, United Nations Globally Harmonized System. Refer to the legend for Figure 19.1 for an aid to interpreting the values of this figure. GHS Category 5 can include chemicals with LC_{50} s in the range equivalent to an oral/dermal LD_{50} between 2000 and 5000 mg/kg. Refer to United Nations (2011) for specifics. (Adapted from Schurger, M.G. and McConnell, F., *Eastman Chemicals*, Kingsport, TN, 1989.)

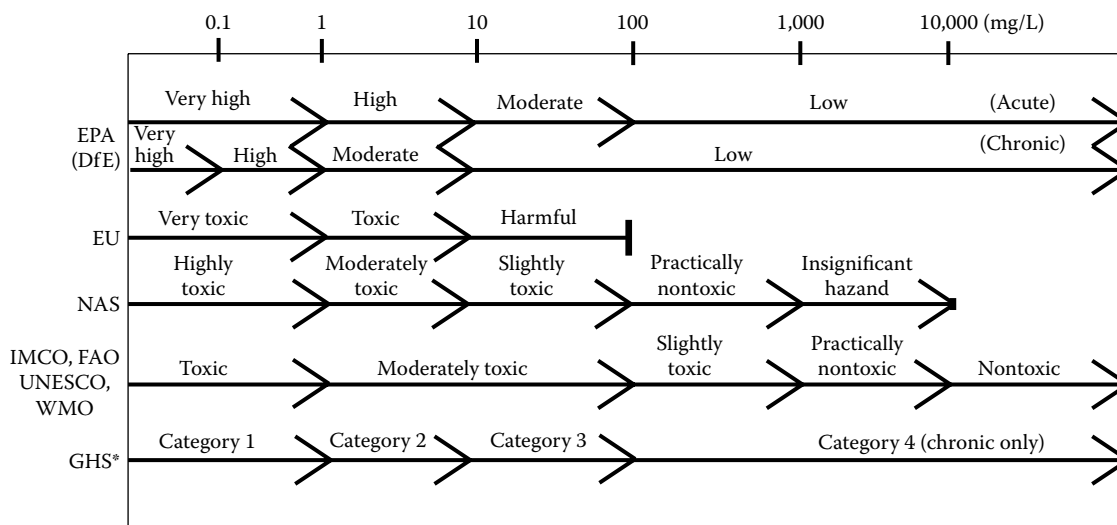


FIGURE 19.5 Classification based on fish acute LC_{50} . *The GHS classification also applies to chronic toxicity provided certain criteria related to biodegradation and bioconcentration are met. Refer to United Nations (2011) for specifics. EPA (DfE), U.S. Environmental Protection Agency (Design for the Environment Program); EU, European Union; NAS, U.S. National Academy of Sciences; IMCO, Intergovernment Maritime Consultative Organization; FAO, Food and Agriculture Organization; UNESCO, United Nations Educational, Scientific and Cultural Organization; WMO, World Meteorological Organization; GHS, United Nations Globally Harmonized System. Refer to the legend for Figure 19.1 for an aid to interpreting the values of this figure. (Adapted from Schurger, M.G. and McConnell, F., *Eastman Chemicals*, Kingsport, TN, 1989.)

TABLE 19.8
Classification Based on Repeated Dose Target Organ Toxicity

	Category	Classification	Criteria			
			NOAEL or LOAEL (mg/kg/day or *mg/L/6 h/day)			
				90 days	40–50 days	28 days
U.S. Environmental Protection Agency Design for the Environment Program (2011)	High		Oral	<10	<20	<30
			Dermal	<20	<40	<60
			Inhalation*			
			Vapor/gas	<0.2	<0.4	<0.6
			Dust/mist/fume	<0.02	<0.04	<0.06
	Moderate		Oral	10–100	20–200	30–300
			Dermal	20–200	40–400	60–600
			Inhalation*			
			Vapor/gas	0.2–1.0	0.4–2.0	0.6–3.0
			Dust/mist/fume	0.02–0.2	0.04–0.4	0.06–0.6
	Low		Oral	>100	>200	>300
			Dermal	>200	>400	>600
			Inhalation*			
			Vapor/gas	>1.0	>2.0	>3.0
			Dust/mist/fume	>0.2	>0.4	>0.6
United Nations Globally Harmonized System (GHS)	1	Significant toxicity in humans	Reliable, good quality human case studies or epidemiological studies			
		Presumed significant toxicity in humans	Animal studies with significant and/or severe toxic effects relevant to humans at generally low exposure (as per guidance values)			
	2	Presumed to be harmful to human health	Animal studies with significant toxic effects relevant to humans at generally moderate exposure (as per guidance values)			
			Human evidence in exceptional cases			

Sources: U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention & Toxics, U.S. Environmental Protection Agency, August 2011 and United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.

TABLE 19.9
Classification Based on Reproductive and Developmental Toxicity

	Category	Classification	Criteria	
			NOAEL or LOAEL (mg/kg/day or *mg/L/day)	
U.S. Environmental Protection Agency Design for the Environment Program (2011)	High	High	Oral	<50
			Dermal	<100
			Inhalation*	
			Vapor/gas	<1
			Dust/mist/fume	<0.1
	Moderate	Moderate	Oral	50–100
			Dermal	100–500
			Inhalation*	
			Vapor/gas	1–2.5
			Dust/mist/fume	0.1–0.5
	Low	Low	Oral	>250–1000
			Dermal	>500–2000
			Inhalation*	
			Vapor/gas	>2.5–20
			Dust/mist/fume	>0.5–5
	Very low	Very low	Oral	>1000
			Dermal	>2000
			Inhalation*	
			Vapor/gas	>20
			Dust/mist/fume	>5
United Nations Globally Harmonized System (GHS)	1A	Known	Known to cause effects on human reproduction or on development based on human evidence	
	1B	Presumed	Presumed to cause effects on human reproduction or on development based on experimental animals	
	2	Suspected	Suspected based on human or animal evidence possibly with other information	
	Additional category		Effects on or via lactation	

Sources: U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention & Toxics, U.S. Environmental Protection Agency, August 2011 and United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.

TABLE 19.10
Classification Based on Eye and Skin Irritation/Corrosivity

	Category	Classification	Criteria
U.S. Environmental Protection Agency Design for the Environment Program (2011)	Eye irritation/corrosivity	Very high	Irritation persists for >21 days or corrosive
		High	Clearing in 8–21 days, severely irritating
		Moderate	Clearing in 7 days or less, moderately irritating
		Low	Clearing in <24 h, mildly irritating
		Very low	Not irritating
	Skin irritation/corrosivity	Very high	Corrosive
		High	Severe irritation at 72 h
		Moderate	Moderate irritation at 72 h
		Low	Mild or slight irritation at 72 h
		Very low	Not irritating
United Nations Globally Harmonized System (GHS)	Eye	1	Irreversible effects on the eye
			At least in one animal effects on the cornea, iris, or conjunctiva that are not expected to reverse or have not fully reversed within an observation period of normally 21 days and/or at least in 2 of 3 animals, a positive response of corneal opacity ≥ 3 and/or iritis ≥ 1.5 calculated as the mean scores following grading at 24, 48, and 72 h after installation of the test material. Category 1 includes other severe reactions (e.g., destruction of cornea)
	2A	Irritating to eyes	At least in 2 of 3 animals a positive response of corneal opacity ≥ 1 and/or conjunctival redness ≥ 2 and/or conjunctival edema (chemosis) ≥ 2 calculated as the mean scores following grading at 24, 48, and 72 h after installation of the test material which fully reverses within an observation period of normally 21 days
			As for Category 2A when the listed effects are fully reversible within 7 days of observation
	Skin	1A	Corrosive
			Corrosive in ≥ 1 of 3 animals ≤ 3 min exposure ≤ 1 hour observation
			>3 min ≤ 1 hour exposure ≤ 14 days observation
			>1 hour ≤ 4 hour exposure ≤ 14 days observation
	2	Irritant	Mean value of $\geq 2.3 \leq 4.0$ for erythema/eschar or for edema in at least 2 of 3 tested animals from gradings at 24, 48 and 72 hours after patch removal or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions (or) Inflammation that persists to the end of the observation period normally 14 days in at least 2 animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling (or) In some cases where there is pronounced variability of response among animals, with very definitive positive effects related to chemical exposure in a single animal but less than the criteria above.
	3	Mild Irritant	Mean value of $\geq 1.5 < 2.3$ 0 for erythema/eschar or for edema in at least 2 of 3 tested animals from grades at 24, 48 and 72 hours or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions (when not included in the irritant category above)

Sources: U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, August 2011 and United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.

TABLE 19.11
Classification Based on Skin and Respiratory Sensitization

	Category	Classification	Criteria
U.S. Environmental Protection Agency Design for the Environment Program (2011)	Skin sensitization	High	High frequency of sensitization in humans and/or high potency in animals ^a
		Moderate	Low to moderate frequency of sensitization in human and/or low to moderate potency in animals ^b
	Respiratory sensitization	Low	Adequate data available but not high or moderate category
		High	Occurrence in humans or evidence of sensitization in humans based on animal or other tests
		Moderate	Limited evidence including the presence of structural alerts
United Nations Globally Harmonized System (GHS)		Low	Adequate data available indicating lack of respiratory sensitization
		Skin sensitizer	A substance is classified as a skin sensitizer if there is evidence in humans that the substance can lead to sensitization by skin contact in a substantial number of persons or if there are positive results from an appropriate animal test
	1A		Substances showing a high frequency of occurrence in humans and/or a high potency in animals can be presumed to have the potential to produce significant sensitization in humans. Severity of reaction may also be considered ^a
	1B		Substances showing a low to moderate occurrence in humans and/or a low to moderate potency in animals can be presumed to have the potential to produce sensitization in humans. Severity of reaction may also be considered ^b
United Nations Globally Harmonized System (GHS)		Respiratory sensitizer	A substance is classified as a respiratory sensitizer if there is evidence in humans that the substance can lead to specific respiratory hypersensitivity and/or if there are positive results from an appropriate animal test
	1A		Substances showing a high frequency of occurrence in humans, or a probability of occurrence of a high sensitization rate in humans based on animal or other tests. Severity of reaction may also be considered
	1B		Substances showing a low to moderate occurrence in humans or a probability of occurrence of a low to moderate sensitization rate in humans based on animal or other tests. Severity of reaction may also be considered

Sources: U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention & Toxics, U.S. Environmental Protection Agency, August 2011 and United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.

^a Human: Positive response $\leq 500 \mu\text{g}/\text{cm}^2$ human patch test induction threshold; Animal: LLNA EC3 value $\leq 2\%$; GPMT $\geq 30\%$ responding at $\leq 0.1\%$ intradermal induction dose or $\geq 60\%$ responding at $>0.1\% - \leq 1\%$ intradermal induction dose; Buehler assay $\geq 15\%$ responding at $\leq 0.2\%$ topical induction dose or $\geq 60\%$ responding at $>0.2\% - \leq 20\%$ topical induction dose.

^b Human: Positive response $> 500 \mu\text{g}/\text{cm}^2$ human patch test induction threshold; Animal: LLNA EC3 value $> 2\%$; GPMT $\geq 30\% - < 60\%$ responding at $>0.1\% - < 1\%$ intradermal induction dose or $\geq 30\%$ responding at $>1\%$ intradermal induction dose; Buehler assay $\geq 15\% - < 60\%$ responding at $>0.2\% - \leq 20\%$ topical induction dose or $\geq 15\%$ responding at $>20\%$ topical induction dose.

TABLE 19.12
Classification Based on Mutagenicity/Genotoxicity

	Category	Classification	Criteria
U.S. Environmental Protection Agency Design for the Environment Program (2011)		Very high	Substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans
		High	Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans OR Evidence of mutagenicity supported by positive results in <i>in vitro</i> AND <i>in vivo</i> somatic cells and/or germ cells of humans or animals
		Moderate	Evidence of mutagenicity supported by positive results in <i>in vitro</i> OR <i>in vivo</i> somatic cells and/or germ cells of humans or animals
		Low	Negative for chromosomal aberrations and gene mutations, or no structural alerts
United Nations Globally Harmonized System (GHS)	1A	Known/presumed	Positive evidence from epidemiological studies
	1B	Known/presumed	Positive results in <i>In vivo</i> heritable germ cell tests in mammals Human germ cell tests <i>In vivo</i> somatic mutagenicity tests combined with some evidence of germ cell mutagenicity
	2	Suspected/possible	May include heritable mutations in human germ cells Positive evidence from tests in mammals and somatic cell tests <i>In vivo</i> somatic genotoxicity supported by <i>in vitro</i> mutagenicity

Sources: U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention & Toxics, U.S. Environmental Protection Agency, August 2011 and United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.

TABLE 19.13
Classification Based on Carcinogenicity

Agency	Category	Classification	Description
U.S. Environmental Protection Agency (2005)		Carcinogenic to humans	This descriptor indicates strong evidence of human carcinogenicity based on epidemiology and experimental information including exposure and mode of action associations
		Likely to be carcinogenic to humans	This descriptor is appropriate when the weight of evidence is adequate to demonstrate carcinogenic potential to humans but does not reach the weight of evidence for the above descriptor <i>Carcinogenic to humans</i>
		Suggestive evidence of carcinogenic potential	This descriptor is appropriate when the available information is suggestive of carcinogenicity and a concern for potential carcinogenic effects in humans is raised, but the information is judged not sufficient for a stronger conclusion
		Inadequate information to assess carcinogenic potential	This descriptor is appropriate when available information is judged inadequate for applying one of the other descriptors
		Not likely to be carcinogenic to humans	This descriptor is appropriate when the available information is considered robust for deciding that there is no basis for human hazard concern
U.S. Environmental Protection Agency (1986)	A	Carcinogenic to humans	Sufficient evidence from epidemiology studies to support a causal association
	B1	Probably carcinogenic to humans	Limited evidence in humans from epidemiology studies
	B2	Probably carcinogenic to humans	Sufficient evidence from animal studies but inadequate or no data in humans
	C	Possibly carcinogenic to humans	Limited or equivocal evidence from animal studies but inadequate or no data in humans
	D	Not classifiable as to humans carcinogenicity	Inadequate or no data from animals and inadequate or no data in humans
	E	Evidence of noncarcinogenicity for humans	No evidence of carcinogenicity in at least two animal species and no evidence in humans
U.S. Environmental Protection Agency Design for the Environment Program (2011)	Very high	Known or presumed human carcinogen	
	High	Suspected human carcinogen	
	Moderate	Limited or marginal evidence of carcinogenicity in animals (and inadequate evidence in humans)	
	Low	Negative studies or robust mechanism-based SAR	Robust mechanism-based SAR analysis which may include (i) negative studies on relevant/suitable analog(s) and/or (ii) combination of lack of structural alerts and features suggestive of potential carcinogenic activity and negative supportive, short-term predictive tests
International Agency for Research on Cancer (IARC)	1	Carcinogenic to humans	Sufficient epidemiological evidence for carcinogenicity in humans or sufficient evidence of carcinogenicity from animals studies with strong evidence for a carcinogenic mechanism relevant to humans
	2A	Probably carcinogenic to humans	Sufficient evidence from animal studies and limited evidence in humans

TABLE 19.13 (continued)
Classification Based on Carcinogenicity

Agency	Category	Classification	Description
European Economic Community (EEC)	2B	Possibly carcinogenic to humans	Sufficient evidence from animal studies but inadequate evidence in humans or limited evidence in humans and less than sufficient evidence in animals
	3	Not classifiable as to human carcinogenicity	Inadequate data to classify
	4	Not carcinogenic	Sufficient evidence of noncarcinogenicity in humans and/or animals
	1	Known to be carcinogenic to humans	Sufficient evidence to establish a causal association between human exposure and cancer
	2	Regarded as if carcinogenic to humans	Sufficient evidence to provide a strong presumption that human exposure may result in cancer. Based on long-term animal studies and/or other relevant information
	3	Causes concern due to possible carcinogenic effects	Inadequate information to make a satisfactory assessment. Some evidence from animal studies but insufficient to place in Category 2
United Nations Globally Harmonized System (GHS)	1A	Known human carcinogen	Based on human evidence
	1B	Presumed human carcinogen	Based on demonstrated animal carcinogenicity
	2	Suspected carcinogen	Limited evidence of human or animal carcinogenicity

Sources: Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chap. 2.; European Economic Community (EEC), 18th Adaptation to Technical Progress, Directive 93/21/EEC, *Off. J. Europ. Econ. Commun.*, 36, No. L110A/61, May 5, 1993; U.S. Environmental Protection Agency, Evaluating pesticides for carcinogenic potential, U.S. Environmental Protection Agency, www.epa.gov/pesticides/health/cancerfs.htm, 2007; U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention & Toxics, U.S. Environmental Protection Agency, August 2011; and United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.

REFERENCES

1. United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, fourth revision edition, New York and Geneva, 2011.
2. Schurger, M.G. and McConnell, F., *Eastman Chemicals*, Kingsport, TN, 1989.
3. U.S. Environmental Protection Agency, Reported in *Pesticide and Toxic Chemical News*, 19, 34, October 1988.
4. U.S. Environmental Protection Agency, *TSCA New Chemicals Program (NCP) Chemical Categories*, Office of Pollution Prevention and Toxics, August 2010.
5. Hodge, H.C. and Sterner, J.H., *Am. Ind. Hygiene Assoc. Q.*, 10, 4, 1949.
6. U.S. Environmental Protection Agency, *Design for the Environment Program, Alternatives Assessment Criteria for Hazard Evaluation*, Version 2.0, Office of Pollution Prevention and Toxics, August 2011.
7. Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chap. 2.
8. European Economic Community (EEC), 18th Adaptation to Technical Progress, Directive 93/21/EEC, *Off. J. Eur. Econ. Commun.*, 36, No. L110A/61, May 5, 1993.
9. U.S. Environmental Protection Agency, Evaluating pesticides for carcinogenic potential, www.epa.gov/pesticides/health/cancerfs.htm, 2007.

20 Pharmaceutical Toxicology

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INTRODUCTION AND THE PHARMACEUTICAL MARKET

The fascinating challenge of drug discovery and drug development to the toxicologist is that drugs are designed to have a specific biological function(s) that provides efficacy in human disease but which maintains a positive risk–benefit profile for the patient. Thus, drug development is necessarily based on an informed, scientific, and (ideally) a mechanistic understanding of toxicity and efficacy. Furthermore, the set of nonclinical studies which support development of a drug are specifically tailored to support clinical trials for a particular treatment regimen, disease indication, and patient population. Pharmaceutical research and development (R&D) represents a somewhat specialized field of R&D in that effects in humans are studied directly using careful and controlled study designs. Therefore, clinical trial results are well suited to evaluate concordance between animal models and human subjects.

Drug development proceeds through a series of generally well-defined phases with carefully considered decision points, and toxicology plays a role throughout that process (Figure 20.1). Predictive knowledge of safety (and efficacy) is typically built starting with *in silico* and *in vitro* screening paradigms and short-term screening studies in animal

models. Study designs are often augmented with parameters to probe mechanistic pathways and include evaluations of exposure and metabolism (“ADME”). Exactly what special assessments are included in more traditional testing designs will vary depending on experience with a therapeutic class or chemical platform, available technology, and value realized in past experience.

The world market for drugs is large and growing. At the end of 2011, global sales of pharmaceuticals topped \$950 billion. The United States, Canada, EU5,* and Japan account for almost 85% of pharmaceutical sales with the balance of the market spread across the rest of the world (ROW).¹ With the consolidation of drug companies and the emergence of small pharmaceutical enterprises, the face of the pharmaceutical industry continues to evolve. The pharmaceutical industry is highly regulated, and the global regulatory landscape is ever-evolving. There has been good progress in harmonizing regulatory requirements across geographies; however, individual countries and regions continue to have unique regulations and guidance that drug companies must follow to gain product approval in those regions. Although the larger markets are often the first that are targeted for regulatory submission and

* United Kingdom, Spain, Germany, France, and Italy.

approval, companies must be sure to consider regulatory requirements of other regions, in particular those of the “Pharmerging” markets such as India, South America, and China. These markets are expected to expand significantly over the next 5 years and potentially outpace the growth in the more traditional markets. Typically, a first-to-market drug captures a major portion of sales, while the successive entries in a drug class fight to develop a market presence. Regardless of the geographic region and the associated challenges, successful regulatory strategies integrate the goal to be “first or best in class.”

The global 10 branded pharmaceuticals for 2011, which accounted for ~8.5% of the total worldwide sales, are presented in Table 20.1. This list will undergo dramatic changes over the next few years due to patent expirations, new technological advances, and the potential for new competition from biosimilars. The therapeutic areas that have seen the greatest development have been those encompassing large populations and chronic diseases and resulted in the model of the billion dollar (USD) “blockbuster” drug.

The business of pharmaceutical development and marketing is high risk and has become fiercely competitive. The industry spends millions of dollars on development of new drugs, although it is well known that the chance of any single candidate reaching the marketplace is extremely low. Overall, it has been estimated that for every 5,000–10,000 molecules, on average only one successfully reaches the consumer market as a drug,^{2,3} and the probability of that new drug entering the market is highly dependent on the therapeutic class.^{4,5} Therefore, industry proceeds with some caution as it pursues development and branches into new classes of drugs. Companies will often invest a great deal of capital in early rapid screening technologies to better eliminate those compounds that show limited promise. With the advent of “omics” technologies and emphasis on the development of biomarkers of disease, the hope is to drive a more efficient, targeted approach to the development of “tailored therapeutics” designed to identify the “right drug” for the “right patient population.” Indeed, the development of pharmacogenomics has led to the possibility, as yet unrealized, of personalized medicine and the development of drugs and treatments for targeted subpopulations. Regardless of these advances, early-stage drug candidates will still drop out of the development process for a variety of reasons, often related to toxicity discovered during the preclinical phase or within the early clinical program. By 2000 in later-stage clinical development, attrition of molecules from further development was most often due to lack of efficacy with ~30% of candidate

drugs being terminated from further development. Safety and toxicology accounted for another 30% attrition.⁶ This trend appears to continue currently although economics plays an increasingly larger role in the choice to discontinue developing a drug or biological candidate. This latter scenario is common with small pharmaceutical enterprises that are dependent on venture capital and other sources of sometimes vicarious external funding to fuel their development activities.

Studies performed to meet regulatory requirements for nonclinical safety assessment can be thought of as belonging to three major categories:

1. Those necessary to support the successful filing/opening of an Investigations New Drug (IND) application, a Clinical Trial Authorization or Notification (CTA, CTN), or equivalent application to support subsequent first-in-human (FIH) (Phase 1) clinical studies.* Such studies include safety pharmacology studies, short-term, repeated dose toxicity studies, and genetic toxicity studies.
2. Those required to support more extensive clinical evaluation in larger (patient) populations (Phase 2 and Phase 3 trials), including women. Such studies include longer-duration repeated dose and reproductive toxicity studies.
3. Those studies required to support a successful marketing approval application. This group of non-clinical studies typically includes pre- and postnatal reproduction and carcinogenicity studies.

A typical sequence of events and studies is reflected in Figures 20.2 and 20.3. Which studies fit into what category is somewhat fluid and heavily driven by the clinical program. For biotechnology-derived products (biologics), the same principles apply although the nonclinical program can be quite different, for example, lifetime rodent bioassays may not be needed for the carcinogenicity assessment. Furthermore, some small molecules follow a different pathway because of the intended indication, for example, oncolytics.

* For this chapter, the term “IND” will be used as the example of an initial submission. In Europe, for example, the comparable document is submitted as a Clinical Trial Application (CTA). In addition, the term New Drug Application (“NDA”) will be used although the comparable terms in Europe and Canada are the Marketing Authorization Application (“MAA”) and New Drug Submission (“NDS”), respectively.

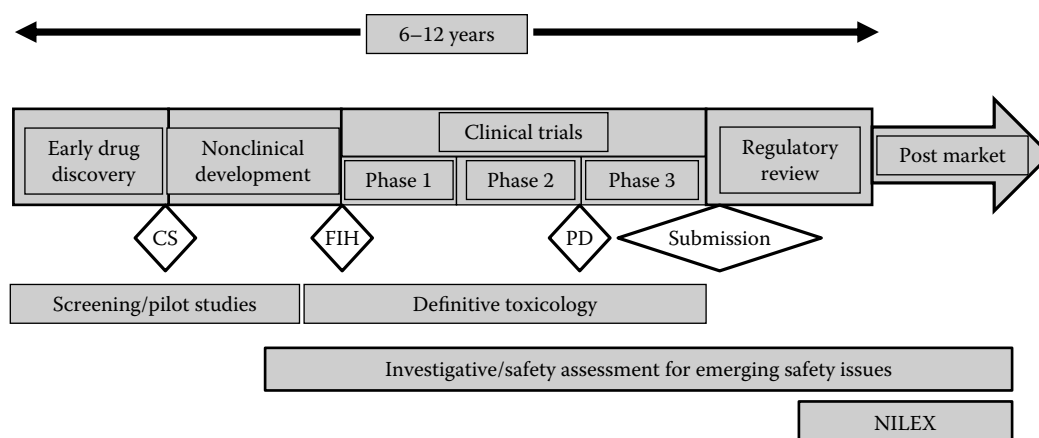


FIGURE 20.1 Toxicology supports the entire drug development process. CS, Candidate Selection; FIH, First in Human; PD, Product Decision; NILEX, New Indications/Line Extensions.

TABLE 20.1
2011 Top 10 Global Pharmaceuticals by Sales

Rank	Medicine	Company	Primary Medical Use	2011 Sales (US\$, Billion)	Percent Growth versus 2010
1	Lipitor	Pfizer	Cholesterol	12.5	-3.3
2	Plavix/Iscover	Bristol-Myers Squibb, Sanofi	Thrombotic events	9.3	3.7
3	Advair/Seretide	GlaxoSmithKline	Asthma	8.7	0.04
4	Crestor	AstraZeneca	Cholesterol	8.0	14.4
5	Nexium	AstraZeneca	Gastrointestinal disorders	7.9	-6.2
6	Seroquel	AstraZeneca, Astellas Pharmaceuticals	Schizophrenia	7.6	9.5
7	Humira	Abbott	Rheumatoid arthritis	7.3	17.8
8	Enbrel	Amgen, Pfizer	Rheumatoid arthritis	6.8	6.7
9	Remicade	Johnson & Johnson, Merck, Tanabe	Rheumatoid arthritis	6.8	8.4
10	Abilify	Otsuka	Schizophrenia	6.3	14.3

Source: Adapted from IMS, Top-Line Market Data, Top 20 Global Products, 2011, 2012, <http://www.imshealth.com/portal/site/ims/> (accessed on July, 2012).

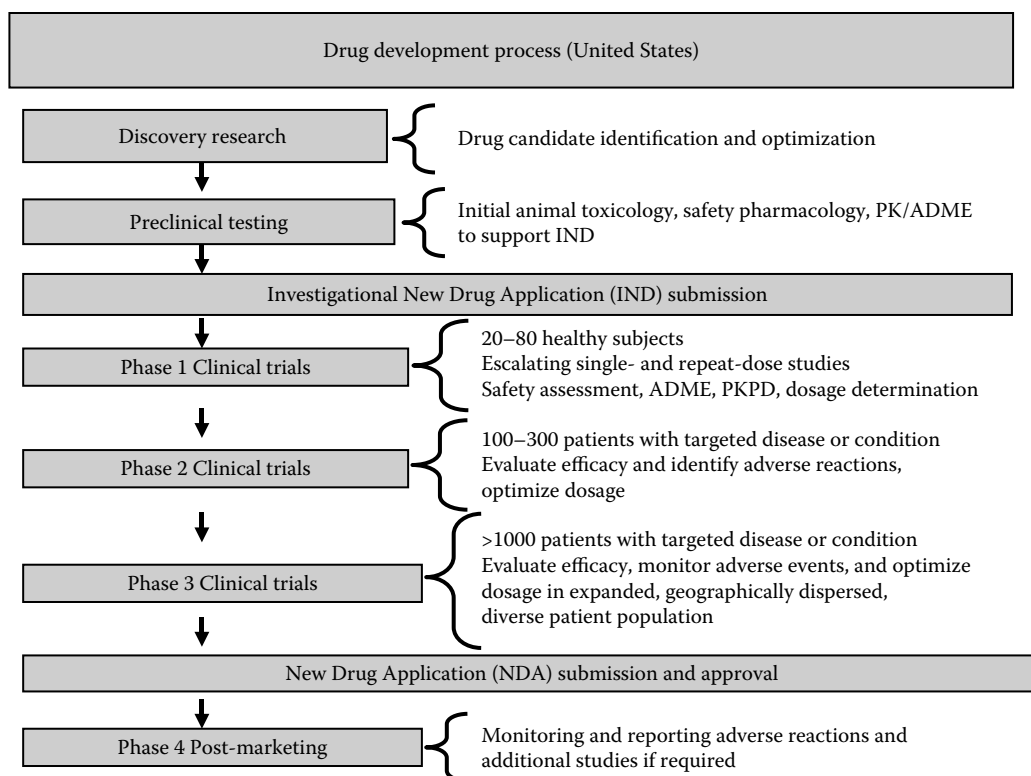


FIGURE 20.2 The drug development process and submission in the United States.

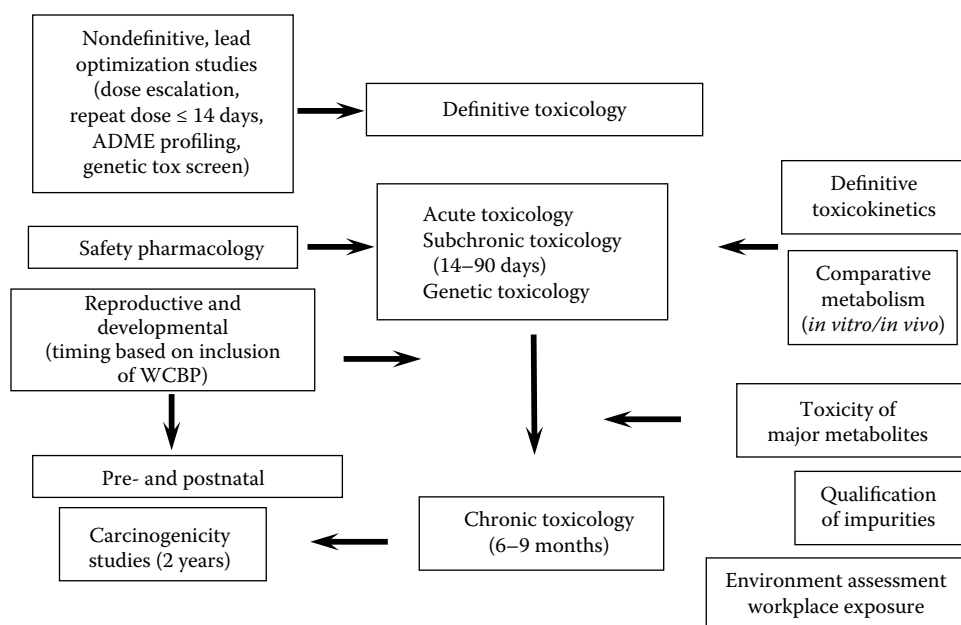


FIGURE 20.3 Studies performed to meet regulatory requirements for nonclinical safety assessment of drugs. (From Dorato, M.A., McMillian, C.L., and Vodicnik, M.J., The toxicologic assessment of pharmaceutical and biotechnology products, In: *Principles and Methods of Toxicology*, 5th edn., A.W. Hayes (ed.), CRC Press, New York, pp. 325–368, 2008.)

INTERNATIONAL REGULATORY AUTHORITIES AND ICH

In the United States, the Food and Drug Administration (FDA) enforces the regulations for approval and withdrawal of drugs and has the regulatory authority for the approval of nonprescription substances, that is, over-the-counter (OTC) substances. This chapter will focus on therapeutic substances (drugs) that require a physician's prescription. According to FDA regulations, a drug is defined as "articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals" (FD&C Title 21, p. 31).

FDA approval of human drugs occurs within the Office of New Drugs (OND) in the Centers for Drug Evaluation and Research (CDER) and for Biologics Evaluation and Research (CBER). Within CDER, there are several offices and divisions with responsibility for specific therapeutic areas, for example, antivirals, oncology, cardiorenal, etc. Similarly, within CBER there are various offices with responsibility for different indications, for example, gene therapy, some vaccines, blood products, etc. (see website links). In CDER, the final drug submission package for small molecules is termed the New Drug Application (NDA). The comparable submission package for biological drugs is called the Biologics License Agreement (BLA).

In Europe, the European Medicines Agency (EMA) has overall responsibility for drug approval. In contrast to the FDA definition of a drug, the EMA definition is two part⁷:

1. Any substance or combination of substances presented as having properties for treating or preventing disease in human beings
2. Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting, or modifying physiological functions by exerting a pharmacological, immunological, or metabolic action, or to making a medical diagnosis

For drug approval in Europe, there are two approaches to pharmaceutical regulation:

1. Use of the *Centralized (or Community) authorization procedure* is obligatory for biological products, orphan drugs, and those intended for treatment of HIV/AIDS, autoimmune diseases and other immune dysfunctions, cancer, diabetes, and neurodegenerative disorders. This procedure has the advantage that it provides a marketing authorization that is valid in all 27 European Union (EU) member states as well as in Iceland, Liechtenstein, and Norway. This process is described in Annex I of Regulation (EC) No. 726/2004.⁷ Generally, most drug approvals occur through the centralized process.
2. The *National and Mutual Recognition procedures* can be subdivided into a decentralized procedure and a mutual recognition (MR) procedure.

The decentralized procedure differs from the MR process based on the momentum of the formal decision. The MR procedure is based upon the decision of one member state, which is to be "mutually" recognized by others. The decentralized procedure is led by one country, but the decision is made after having the input from all the contributing European member states, but not necessarily including all European countries.

Much of the scientific work for drug approval in Europe is undertaken by six committees:

1. Committee for Medicinal Products for Human Use (CHMP)
2. Committee for Medicinal Products for Veterinary Use (CVMP)
3. Committee for Orphan Medicinal Products (COMP)
4. Committee on Herbal Medicinal Products (HMPC)
5. Paediatric Committee (PDCO)
6. Committee on Advanced Therapies (CAT)

Of these, CHMP, PDCO, and COMP are especially important committees for the approval of drugs in Europe.

In Japan, the Ministry of Health, Labor and Welfare (MHLW) has the authority for the approval of drugs. However, applications for drug approval and the initiation of clinical trials are reviewed by the Pharmaceutical and Medical Device Agency (PMDA) within the MHLW. The process for submission, review, and approval are similar to that of the FDA. Indeed, within the PMDA, drug applications are submitted to the OND or to the Office of Biologics. Within these offices, different groups review a drug application based on the proposed therapeutic indication.

The United States, EU, and Japan represent the major international regulatory authorities, although other regions, for example, Australia, Canada, China, Africa, South America, have established regulatory processes for drug approval, and the process across these additional regions generally reflects the process in the United States and Europe.

Compliance with multiple national regulations can be challenging and time-consuming and results in quite varied differences with respect to nonclinical and clinical data requirements. Because of these differences in regulatory processes, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) began in 1988 with support from the three major regulatory regions—Japan, Europe, and the United States. Japan, the United States, and Europe represent the core of ICH regions, but many countries have adopted the ICH process within their regulatory framework. Indeed, Canada, Australia, Russia, and other major regional authorities actively participate in the ICH process for the adoption of new guidelines. It is important to understand that region-specific requirements still exist within some regions, for example, Japan. The ICH is not a regulatory authority, and ICH

Guidelines, when promulgated, convey no *legal* authority. These guidelines are submitted to the appropriate national (or regional) regulatory authorities for official adoption and publication at which time the guideline could convey legal authority in, for example, the EU.

NONCLINICAL TESTING AND REGULATORY SUBMISSIONS

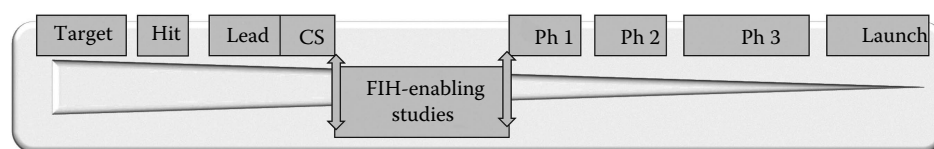
The worldwide regulatory bodies require that the safety and efficacy of a drug be demonstrated prior to approval and, hence, commercialization. For the toxicologist, the design and conduct of nonclinical studies to demonstrate the safety of the drug prior to entry into a clinical trial is of prime importance. The toxicology program should be designed to identify key toxicity signals and end points that could provide useful information for dose administration and safety monitoring in initial clinical studies where the tolerability of the drug is examined (usually in healthy volunteers). An IND submission will include the data tables and narratives of the supporting nonclinical toxicology, pharmacokinetics, and pharmacology studies, and supporting justification for the dose level(s) selected for Phase 1 trials.⁸ It is important that the IND provide a “holistic” view of the data and perspective for the regulatory reviewer, that is, the IND should “tell a story” of the findings and the meaningfulness of the data to support of the intended clinical trials.

The nonclinical studies required to initiate FIH clinical studies of pharmaceuticals are variously called “IND- or FIH-enabling” studies. All the pivotal toxicity studies required for initiation of the clinical study are to be conducted in compliance with Good Laboratory Practice (GLP) regulations, although there can be exceptions, for example, for special or “novel” studies.

In preparation for the IND-enabling studies, there are a variety of items that must be addressed well in advance of drug administration to the first animal in the first pivotal toxicity study (Table 20.2).

The content of the FDA IND is shown in Table 20.3 (21CFR Part 312.23). The most common type of IND submitted by pharmaceutical companies is the commercial IND. Other types of INDs (an Investigator IND, an Emergency IND, a Treatment IND, and a Screening IND) are described more by Mathieu.⁹ Many of the sections identified in Table 20.3 are self-explanatory.

Many sponsors now format their IND submission using that of the Common Technical Document, the document format required for submission of a NDA.¹⁰ Although not consistent with the IND format described earlier, the CTD format has become an acceptable and, in some cases an expected format for submission to the FDA. The CTD is organized into five modules: Module 1 is region-specific, while Modules 2, 3, 4, and 5 are intended to be common for all regions (Table 20.4).



Indication (guidance)	General toxicology Rodent/nonrodent	Genetic toxicology	Safety pharmacology
Non-oncology (ICH M3(R2))	2–4 week daily dosing	Ames, chromosomal Ab, MNT (<i>in vivo</i>)	Cardiovascular, CNS, respiratory
Oncology (ICH S9)	Dosing to cover clinical schedules; reversibility in 1 species	None	No stand-alone studies (combined with toxicity studies)
Biologics (ICH S6(A1))	Dosing to cover Phase 1 schedule and human $t_{1/2}$; may be limited relevant models	None	No stand-alone studies (combined with toxicity studies)

TABLE 20.2
Prestudy Considerations for Nonclinical Studies

Item	Description
Characterization of the drug substance	The sponsor must remember that the purity (small molecules) or characterization (large molecules) of the drug substance used in the clinical studies should be supported by the test material and doses administered in the nonclinical studies. For example, the amount of an impurity present in a human dose must be “covered” (qualified) by at least that amount having been administered in the animal studies. Too often, this basic concept in early-stage development is overlooked and can be cause for a delay in approval of the clinical trial until this or other safety-related issues are addressed by the sponsor.
Development and validation of GLP-compliant analytical methods	These studies are necessary to verify the purity of the drug substance and measure the drug substance in dose formulations.
Development and validation of GLP-compliant bioanalytical methods	These studies are necessary to measure the amount of parent drug, and metabolites if necessary, present in biological fluid (blood, plasma, urine, etc.) of selected test species. Validation needs to include the concentration range anticipated to be present in the nonclinical study, short- and long-term stability of the drug in samples, specificity and accuracy of the method, etc.
Test article characterization (e.g., stability, homogeneity)	Stability of the drug substance under sponsor-specified storage conditions and in the anticipated animal dosing formulation over the expected duration of the study must be demonstrated. For example, if a study is 28 days in duration, the stability of the drug substance should cover this duration plus any time required for shipping, storage, protocol preparation, etc. for the study.

TABLE 20.3
Content of an Investigational New Drug (IND) Application

IND Section No.	Section Title	Description
1	IND cover sheets (Form FDA 1571)	FDA Form 1571 allows the FDA to quickly identify what is being submitted.
2	Table of contents	
3	Introductory statement and general (clinical) investigation plan	A general description of the drug, formulation to be used in the clinical trial, a description of the anticipated pharmaceutical claim and the clinical hypothesis, and description of the clinical study to be undertaken.
4	Reserved	This section can be used by the FDA for any other consideration for the IND.
5	Clinical Investigator’s Brochure (IB)	Written for the clinical investigator, the IB provides an overall description of the clinical and nonclinical safety and efficacy of the drug substance and a description of the chemical and physical properties of the drug substance.
6	Proposed clinical protocol(s)	A detailed description of the proposed clinical study.
7	Chemistry, manufacturing, and control information	Detailed description of chemical and physical properties of the drug substance and drug product formulation, impurity and degradant information, manufacturing information, container closure data, etc.
8	Pharmacology and toxicology information	Description of all toxicology, pharmacology, and pharmacokinetic data. Impurities, degradants, and metabolites are also included.
9	Previous human experience with the investigational drug	Any clinical information that has been generated for the drug substance in studies outside the United States or conducted under a Screening IND (e.g., Phase 0 studies).
10	Additional information	These sections can describe the use of radiolabeled drugs in the clinical trial and a description of the safety of the radiation-absorbed dose, drug dependency, pediatric studies, and an assessment for the use of the drug in pediatric populations, etc.
11	Other relevant information	These sections permit the sponsor to tell the Agency about special circumstances that potentially impacts the approval of the IND.

TABLE 20.4
Content of the Common Technical Document (CTD)

Module Number	Description
1	This module contains documents specific to each region; for example, application forms or the proposed label for use in the region. The content and format of this module can be modified to be consistent with the needs of the regulatory authority.
2	CTD Summaries: Contain the bulk of the nonclinical and clinical information, provided in tabular and narrative form. It should begin with a general introduction to the pharmaceutical, including its pharmacological class, mode of action, and a thorough description of all nonclinical data.
3	Information on the drug product, physiochemical properties, drug formulation information, etc.
4	Nonclinical Study Reports: The nonclinical study reports are to be provided in this section.
5	Clinical Study Reports: If clinical data are available, the study reports and related information are to be included in this section.

NONCLINICAL TESTING: GENERAL CONDITIONS AND CONSIDERATIONS

For the discussion later, a general case approach to nonclinical safety assessment will be considered, supporting daily oral administration of a chronic use drug. Summary discussions of various types of studies are presented. Individual chapters of this book provide greater detail, for example, Chapter 21, Safety Pharmacology. Many aspects of nonclinical safety assessment are not dependent on the route of administration, although the route can impact, for example, animal model selection (e.g., topical drugs), local toxicity, and drug disposition. Generally, the frequency of drug administration (dose regimen) is once daily, though dose regimens may be less frequent, particularly for biological and oncolytic drug products. As it is not possible within this chapter to describe all eventualities of a drug development process—this can be as varied as the number of drugs being investigated—the reader is encouraged to review many of the cited publications and guidance documents.

The IND-enabling studies typically support single and/or repeat dose Phase 1 clinical studies up to 2–4 weeks in duration. Longer-term clinical trials require the support of correspondingly longer-duration nonclinical studies.¹¹ The repeat dose toxicity studies must be conducted in a rodent and a nonrodent species, although there may be exceptions. For example, the nonhuman primate (NHP) may be the only pharmacologically relevant, and therefore appropriate, species for testing highly specific biological products. Additionally, developmental and reproductive toxicity studies (DART) are usually required to allow the inclusion of a broader range of patients (e.g., women of child-bearing potential [WOCBP]) in clinical trials. The longer-term, repeat dose studies are generally conducted in incremental steps (e.g., 1, 3, and 6 month studies) to support Phase 2 and subsequent Phase 3 clinical trials.

The last distinct part of the nonclinical safety assessment study package generally consists of studies which are not required until the NDA is submitted. This group of studies is usually limited to carcinogenicity studies (if required) and the final parts of the reproductive toxicity

package, although some special requirements may arise in specific situations including impurity qualification, metabolite testing, detailed studies of mechanism of drug action or toxicity, etc.

The new drug substance (NDS) and new drug product (NDP) used in humans must be qualified in pivotal toxicology studies. This assessment includes the active drug ingredient (API) as well as impurities, residual solvents, and excipients (the latter elements may be qualified based on safety data in the literature).¹² However, the manufacturing process and site may change several times over the course of clinical development, such that it is important that later-phase toxicology studies use the final NDS or the nonclinical studies may need to be repeated, that is, bridging studies. Generally, bridging studies are usually 4 week studies in the more sensitive animal model; genotoxicity and an embryo–fetal development (EFD) study may also be needed.

For routes of administration other than oral, additional studies must address issues of local tissue response to the administered clinical drug product. For intravenous drug products, for example, a test for hemolysis, intravenous/extravascular irritation, and protein precipitation are often required. Sensitization and phototoxicity studies will be needed for dermal products. Similarly, route-specific irritation assays (eye, skin, muscle, mucosal, etc.) are needed for clinical trials as well as for workplace exposure guidelines.

As noted previously, testing of pharmaceuticals usually proceeds in a rather fixed and orderly way. The duration of the repeat dose nonclinical studies generally will need to mirror the duration of the clinical trial (Table 20.5). As noted previously, this listing does not account for all possibilities during a drug development program. In spite of the recommendations provided in Table 20.5, there are multiple factors associated with duration of a nonclinical study relative to the duration of the clinical trial which includes patient population, indication, large versus small molecule drug substance, etc.¹¹

As the duration of the clinical study increases, the duration of the supporting nonclinical studies will also need to increase in duration. It is possible to initiate a single dose

clinical study for some pharmaceuticals by providing data from a single dose toxicity study in two species. The ICH M3(R2) guideline outlines several scenarios (e.g., species, doses, safety end points) whereby different nonclinical studies may support very early clinical studies of limited scope.¹¹ These studies must include clinical pathology and histopathology as well as be fully GLP-compliant.

Another aspect of undertaking the nonclinical study is high-dose selection. Although this topic is beyond the scope of this chapter, dose level selection is a principal factor in the success, and failure, of a nonclinical study intended to support a clinical trial. The ICH M3(R2)¹¹ guideline provides a general guide for dose level selection. The high dose should result in toxicity, preferably non-life-threatening toxicity (e.g., a maximum tolerated dose, MTD) with a graded response observed at lower doses. The low dose should be a clear no observable adverse effect level (NOAEL).¹³

Table 20.6 presents general guidance on the least number of animals to be used in pivotal toxicity studies supporting clinical development and regulatory submission. The numbers shown in Table 20.6 usually represent the minimum numbers required, and is based on Organization for Economic Cooperation and Development (OECD) guidelines (see website link). Indeed, many of the guidelines and guidance developed for pharmaceutical testing are derived from the OECD guidelines. Ten rats/sex/group are often used for 2 and 4 week studies. Additional rodents are needed for the collection of blood samples for toxicokinetic determinations. The number of nonrodents, for example, dogs or NHPs, generally has been 3/sex/group for 2 and 4 week studies, although some regulatory authorities have requested more animals per group for the treatment phase of the study. Additional animals will be needed to examine the reversibility of potential effects observed during the treatment phase of the study; inclusion of a recovery phase in at least one repeat dose study in rodents and in nonrodents is standard.

Typical end points included in a repeat dose study protocol are outlined in the OECD guidelines and include measurement of body weights, recording of clinical observations, as well as daily observations for mortality and morbidity; hematology and clinical chemical parameters; and, at necropsy, organ weights, gross, and microscopic pathology of tissues. Ophthalmological examinations need to be done in pivotal studies, especially for rabbits and dogs. Furthermore, for drugs that target the CNS, for example, antipsychotics, special staining of neural structures should be incorporated into the study.^{14–16}

In recent years, there has been increased use of electrocardiograms (ECG) in nonrodent species during the conduct of pivotal repeat dose studies.¹⁷ Although this continues to be debated in the toxicology community, these end points have become so common that it is advisable to continue to include these end points. Furthermore, ICH S2(R1)¹⁸ provides options for incorporating genotoxicity end points in repeat dose studies.

TABLE 20.5
General Guidelines for Animal Toxicity Studies in Early Development

Maximum Duration of Clinical Trial	Recommended Duration of Repeat Dose Toxicity Studies	
	Rodents	Nonrodents
Up to 2 weeks	2 weeks	2 weeks
2 weeks through 6 months	Same as clinical trial	Same as clinical trial
>6 months	6 months	9 months

Source: International Conference on Harmonization (ICH), Guidance on the non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, M3(R2), 2009.

TABLE 20.6
Numbers of Animals per Dose Group

Study Duration (per Sex)	Rodents (per Sex)	Nonrodents (per Sex)
2–4 weeks	5–10	4
13 weeks	20	6
26 weeks	30	8
Chronic	50	10

SAFETY PHARMACOLOGY STUDIES

Safety pharmacology studies are typically included with the IND for a Phase 1 clinical study.¹⁹ The base set of studies consists of assessments of cardiovascular (CVS), respiratory, and central nervous system (CNS) function. Species selection is based on the pharmacological relevance, historical experience with certain animal models, and any special physiological characteristics of the animal model as it relates to humans. In the respiratory and CNS studies, the rat is the preferred species although other species can be used with scientific justification. For the CVS study, the conscious dog is the preferred species, although the NHP has also been used. Over the last few years, a combined study of CVS and respiratory system in the dog or NHP has become more common as this tends to reduce animal usage and the timeline for development.^{17,20} Further, this study design has been found to be acceptable to the regulatory authorities. Development and validation of this combined study in other species, such as the minipig, has also been reported.^{21–23}

Typically, single doses are administered and should result in plasma concentrations (C_{\max}) that provide multiples of the efficacious concentration in humans. The time points for the measurements should be based on optimal pharmacodynamic and pharmacokinetic considerations (e.g., proximity to T_{\max}).

Motor activity, behavioral changes, coordination, sensory/motor reflex responses, and body temperature are assessed in the CNS safety pharmacology study, and the functional observation battery (FOB), a modified Irwin's or other

appropriate test, can be used. In the respiratory safety pharmacology study, respiratory rate, tidal volume, and minute volume should be evaluated. Effects of the test substance on the CVS system should be assessed with heart rate and electrocardiographic measures.²⁴ In conjunction with the *in vitro* hERG assay, the *in vivo* measure of prolongation of the QT interval is of paramount importance in assessing possible risk of torsades de pointe, an often fatal heart arrhythmia.

Although not usually required for Phase 1 studies, other safety pharmacology studies are necessary when the mode of action of the pharmaceutical is expected to cause a safety concern. Drugs intended for renal therapy will require a renal pharmacology study (cytology in the urine, glomerular filtration rate, electrolyte concentrations, etc.). Most often, these studies are conducted with rats although dogs, NHPs, and swine have also been used. If a gastrointestinal pharmacology study is appropriate, propulsion rate and alterations in absorption potential are examined, usually in rats.

Safety pharmacology studies may not be needed for locally applied agents, for example, dermal agents when systemic exposure is demonstrated to be low. Indeed, these studies are often not needed for dermal agents that are intended for topical indications such as acne, rosacea, etc. However, when systemic exposure is expected because of chronic, damaged skin, safety pharmacology studies are usually needed. Safety pharmacology studies for cytotoxic agents used for the treatment of end-stage cancer patients may not be necessary; however, there may be value in conducting safety pharmacology studies with cytotoxic (cytostatic) agents having novel mechanisms of action.

GENOTOXICITY STUDIES

Genetic toxicity testing generally focuses on the potential of a new drug to cause mutations (in single-cell systems) or other forms of genetic damage. The tests, generally short in duration, often rely on *in vitro* systems and generally have a single end point of effect (point mutations, chromosomal damage, etc.). Protocol guidance has been provided by the ICH¹⁸ with more protocol specificity provided by the OECD and in published literature.^{25,26} According to ICH, two options are provided for undertaking genotoxicity studies (Table 20.7). In Option 2, some of the genotoxic end points can be included in repeat dose toxicity studies.

Most sponsors conduct three genotoxicity studies for the IND to assess the potential for bacterial mutation (Ames assay), clastogenicity (e.g., *in vitro* chromosomal aberration), and DNA damage *in vivo* (e.g., the micronucleus test). If equivocal results or clear evidence of genotoxicity is observed, appropriate follow-up studies (e.g., a Comet assay, unscheduled DNA synthesis, etc.) should be performed prior to submission of the IND to clarify the findings and add perspective for human risk assessment. Thus, a positive genotoxicity result does not necessarily lead to termination of development, but the sponsor would be expected to follow up on this positive with additional testing.

In certain cases, genotoxicity testing may not be necessary. Clearly, such studies have little value if the drug being developed is a cytotoxic oncolytic agent, however,

TABLE 20.7
ICH Battery of Genotoxicity Studies

Option 1	Option 2
Bacterial reverse mutation assay	Bacterial reverse mutation assay
<i>In vitro</i> mammalian cell assay to detect chromosomal aberrations of micronuclei	<i>In vivo</i> genotoxicity in rodent hematopoietic cells
<i>In vivo</i> genotoxicity in rodent hematopoietic cells, either micronuclei or chromosomal aberrations	Second <i>in vivo</i> assay/end point in a second tissue, for example, liver

genotoxicity studies may need to be conducted for cytostatic oncology agents. Genotoxicity testing of antibiotics, particularly in bacterial systems, may have limited value. The FDA has requested these studies using *in vitro* concentrations below cytotoxic levels. Finally, for some therapeutics, for example, biological therapeutics, genotoxicity studies are not usually applicable or required.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

Special studies are conducted to evaluate risk of reproductive and developmental toxicity. The ICH S5(R2)²⁷ guideline (Figure 20.4) describes study designs for fertility and early embryonic development, EFD (teratogenicity), and pre- and postnatal development (PPND) which includes maternal function.*

ICH M3(R2)¹¹ provides guidance regarding the timing for conduct of these studies relative to the clinical trials. Studies of fertility and general reproductive performance and EFD (often combined in one study) are generally completed to support Phase 2 studies, particularly if WOCBP are included. These studies may be required for Phase 1 if the drug represents a special risk for reproductive toxicity, for example, estrogenic- or androgen-like actions.

The DART studies are generally conducted in rodents (rats), and EFD testing is required in two species—a rodent (rat or mouse) and the rabbit. On occasion, when a test article is not compatible with the rabbit, EFD data in the mouse may be substituted. Both the fertility/early embryonic development and EFD studies should be completed before entering Phase 3 clinical trials. The more complex PPND protocol is generally commenced during Phase 3 trials and to be submitted as part of the NDA. There are also some specific classes of therapeutics, for example, quinolone antibiotics, where special EFD studies in primates are required prior to product approval.

* Historically, the fertility and general reproductive performance, embryo-fetal development, and the pre- and postnatal studies have been referred to as Segment (Seg) I, II, and III studies, respectively.

Reproductive and development periods					
A: Premating through conception			Fertility/early embryonic (Seg I)		
B: Conception through implantation					
C: Implantation through closure of hard palate			Embryo–fetal (Seg II)		
D: Closure of palate through end of pregnancy					
E: Birth to weaning			PPND (Seg III)		
F: Weaning to sexual maturity					

Species	Implantation (GD)	Closure of hard cleft palate (GD)	Usual parturition (days)	Estrous cycle (days)	Number of offspring
Rat	6	17	21–22	4–6	6–12
Mouse	6	15	19–20	3–9	4–10
Rabbit	7	19	30–32	None	6–9
Human	6–7	~50–56	266	28	1

FIGURE 20.4 Nonclinical studies assessing reproductive and developmental toxicity across the ICH-defined phases are depicted in the upper figure. The lower table provides average reproductive milestone dates and durations for rat, mouse, rabbit, and human. GD, Gestation day; PPND, pre- and postnatal development; Seg, segment.

CARCINOGENICITY STUDIES

All chronic use drugs must be assessed for possible risk of carcinogenicity. Such assessments typically include lifetime bioassays in rats and mice, the practice of which emerged in the 1960s with an increased awareness that chemicals can cause cancer. At the time, the primary focus was on commodity chemicals or those found in the environment and, to lesser extent, with drugs. In the 1990s, the ICH Guidelines for carcinogenicity testing provided specific direction on critical aspects of carcinogenicity study design and specified under what circumstances carcinogenicity testing was required for pharmaceuticals.

The objective of a carcinogenicity test is to identify the carcinogenic potential (e.g., increased tumors) in animals and to assess the relevance to carcinogenic risk in humans. The study must be designed with sufficient power to detect a response, for example, adequate numbers of animals, range of doses, and length of dosing period (lifetime, e.g., 1.5–2 years) and include a full tumor characterization through histopathology. The second component of a carcinogenicity assessment—to assess the relevant risk to humans—is more complex. If a tumor response occurs in the study, this second component generally demands that additional insight into the tumor response be developed beyond the simple identification of a statistically elevated tumor incidence in treated versus control animals. For pharmaceuticals as well as most xenobiotics, a “yes/no” answer to carcinogenicity in animals has very limited value in assessing human risk particularly since most drugs are not genotoxic. The occurrence of the tumors in animals related to defined animal drug exposures is important information to compare to human exposures in assessing risk. If the tumor response is clearly restricted to a high-dose group (e.g., a threshold exposure exists) and the

tumor response occurs at exposures substantially higher than humans receiving therapeutic doses, the concern for human risk is greatly reduced.

The ICH S1A²⁸ guideline outlining the need for carcinogenicity studies clarified the criteria used to determine the requirement for a carcinogenicity study. These criteria include the duration and exposure of human treatment, concern for carcinogenic potential based on a number of scientific parameters related to the drug or related drugs, genotoxicity, the indication and patient population, route of exposure, and extent of systemic exposure. A carcinogenicity study is usually required when the drug is indicated for chronic use, defined as daily administration or for intermittent cycles resulting in exposure equivalent to 6 month duration. Carcinogenicity studies may not be required where the life expectancy of the treated population is less than 2–3 years, for example, late-stage cancer. Consistent with this point, ICH S9 guideline notes that carcinogenicity studies are not required for therapies for advanced cancer.^{29,30}

The ICH S1B guideline provides perspective on approaches for evaluation of carcinogenicity.³¹ This guideline indicates how species should be selected, introduces the acceptability of various subchronic rodent test systems (transgenic animal models) as alternatives to the lifetime rodent bioassay, and discusses the importance and significance of mechanistic studies in the interpretation of carcinogenicity study results.

The final ICH S1C guideline represents a most important aspect of carcinogenicity testing, that is, dose selection.^{32,33} This guideline outlines multiple approaches for the selection of a high dose in addition to the possible use of a MTD which was considered the primary approach for the evaluation of carcinogenicity of pharmaceuticals in the past.

The conduct of range-finding studies (90 day repeat dose studies) is an absolute necessity for appropriate design of the carcinogenicity studies; such range-finding studies should include standard toxicological end points as well as end points that will help in understanding the mechanism of toxicity (satellite groups). The S1CR2 guideline also defines multiple points for consideration in the selection of the mid and low doses in carcinogenicity studies. While this guideline addresses the selection of doses in 2 year carcinogenicity studies, it does not necessarily apply to the design of transgenic animal studies.^{34,35}

Because of the significant cost (1–2 million USD) and importance of these studies in human risk assessment, it is critical that a sponsor submits a carcinogenicity study protocol to the regulatory authority for review prior to initiation of the study. In the United States, review of carcinogenicity study plans by the FDA Carcinogenicity Assessment Committee (CAC) provides an important opportunity for an assessment of the scientific and performance aspects of carcinogenicity studies.³⁶ FDA should evaluate and comment on the proposed protocols within 45 days. Submitted protocols should be complete and provide justification for dose levels, route of administration, end points to be examined including toxicokinetics, and details of pathological examination. Any specific end points to examine a potential mechanism of toxicity should be described in detail with justification. Typically, clinical pathology end points are not included in carcinogenicity bioassays.³⁷ As noted previously, carcinogenicity studies for biologics are not often conducted.³⁸

JUVENILE TOXICITY STUDIES

Recent regulations in the United States and EU (Best Pharmaceuticals for Children's Act; Pediatric Research Equity Act; Medicinal Products for Paediatric Use) have highlighted the need to understand safety and efficacy in pediatric populations even though a drug might not be specifically developed for that population. A safety assessment to support trials in pediatric patients must carefully consider all data available at the time a pediatric clinical program is being planned. It may be that an assessment of the known pharmacology, drug disposition, and safety data support safe entry into clinical pharmacokinetic studies in children, especially if clear biomarkers of effect are available. These data come from the pharmacology studies, ADME (absorption, distribution, metabolism, and excretion) studies, and toxicity studies in adult animals as well as data from adult clinical trials, the literature, and previous knowledge of the drug or chemical class. Data from developmental toxicity studies typically include

embryo–fetal and pre- and postnatal studies in which the offspring are exposed indirectly either *in utero* or postnatally through the milk. While useful, extrapolation of these data to potential effects in juvenile animals is limited as there is no direct exposure to the young animal.

There has been an increasing emphasis on the conduct of juvenile animal toxicity studies which offer an opportunity to address specific questions of potential toxicity in the growing animal not usually assessed in the adult toxicity studies, for example, impaired growth or developmental milestones, prior to the start of pediatric clinical trials. The EMA and the FDA have issued specific guidance for the conduct of these studies, and ICH Guideline M3(R2) also notes the role of the juvenile toxicity study.^{11,39,40} Again, a sponsor is well advised to consult the regulatory authority prior to initiation of these studies. These studies are most often conducted after many of the toxicity studies in adult animals have been completed, and the Phase 3 clinical trials are, at least, in progress if not completed. The design of the study is determined primarily on the specific indication and, more importantly, on the pediatric population to be treated. The designs of the studies are outlined in the guidance mentioned earlier and in a number of publications.^{41–44}

The end points included in juvenile toxicity studies are consistent with those included in adult animal studies.^{45,46} Juvenile animal studies are especially designed to examine aspects of function or development of a particular target organ(s). The organ systems examined routinely are the CNS, skeletal, reproductive, pulmonary, CVS, renal, and immune systems. Study protocols should take into account the pharmacological activity of the drug, the expected target organ or system toxicity, and specific concerns from adult animal toxicity studies and adult human clinical experience. Additional considerations are species, age at start of treatment, study duration, route of administration, dose selection, study end points, sample size, route of exposure, etc. The feasibility of the study also is a consideration. For example, treatment of 4 day old rat pups with a drug that affects fluid balance may not be feasible since the only fluid available to the pup is limited to maternal milk.

Undertaking the juvenile toxicity studies requires an understanding of the comparative postnatal development in animal models and humans. A general guide for comparative age categories between different animal species and humans with regard to CNS and reproductive organ development is presented in Figure 20.5.⁴⁷ In addition, a series of minireviews has been published on the heart,⁴⁸ lung,⁴⁹ kidney,⁵⁰ CNS,^{51,52} immune system,⁵³ male and female reproductive systems,^{54,55} gastrointestinal system,⁵⁶ and bone and postnatal growth.⁵⁷

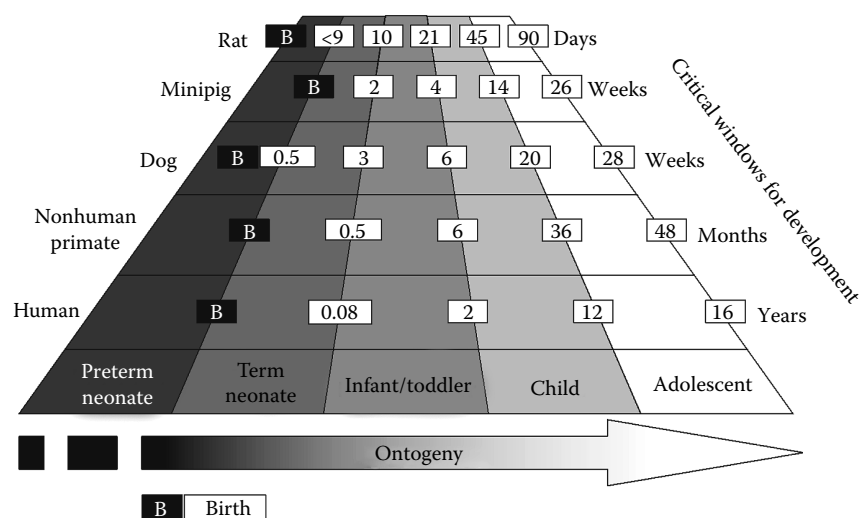


FIGURE 20.5 Comparative age categories across species based on CNS and reproductive development. Estimates are based on combined developmental events occurring in both genders and represent the overall schedule for CNS and reproductive development in these species. The end age of the infant/toddler corresponds roughly to the usual age at weaning for laboratory animals. (From Buelke-Sam, J., Comparative schedules of development in rats and humans: Implications for developmental neurotoxicity testing, *Presented at the Annual Meeting of the Society of Toxicology*, Salt Lake City, UT, 2003.)

TOXICITY TESTING: BIOTECHNOLOGY PRODUCTS

New technology created in the 1980s sparked the development of a new industry—the biotech industry—and an ensuing explosion of new biotherapeutic agents. Products, such as recombinant DNA–produced proteins, biological response modifiers (cytokines and colony-stimulating factors), monoclonal antibodies, antisense oligonucleotides, and self-directed vaccines (raising an immune response to self-proteins such as gastrin for therapeutic reasons), emerged on the market with limited guidance on how to assess and manage the safety and efficacy of these new products. The new bioproducts were distinct from small molecule therapeutics with regard to such characteristics as their highly targeted mechanisms of action, the potential for immunologic response to these large biomolecules, and differences in drug disposition (e.g., nonenzymatic proteolytic degradation, limited off-target tissue binding). Such differential properties raised a variety of questions on the appropriateness of traditional methods of evaluating drug toxicity that generated several points-to-consider documents. Some of the safety issues that arose over the years included

- The appropriateness of testing a human-specific peptide hormone in nonhuman species.
- The potential that the peptide could break down due to nonspecific metabolism resulting in products that had no therapeutic value or even a toxic fragment.

- The potential sequelae to an immune response (formation of neutralizing antibodies, provoking an autoimmune or a hypersensitivity response), and pathology due to immune precipitation, etc.
- The presence of contamination with oncogenic virus DNA (depending on whether a bacterial or mammalian system was used on the synthesizing agent) or endotoxins.
- The difficulty interpreting the scientific relevance of response to supraphysiological systemic doses of potent biological response modifiers.

The regulatory thinking and experience over the last 15 years have come together in the ICH Guidance on testing of biotechnology-derived drugs.⁵⁸ This guidance provides current advice and considerations regarding safety evaluation of biotechnology-derived products and applies to investigational, protein therapeutic, diagnostic, and prophylactic products derived from characterized cells through the use of expression systems such as bacteria, yeast, insect, plant, and mammalian cells and produced by cells in culture or by recombinant DNA technology, including transgenic plants and animals. Many of the principles outlined earlier for small molecules apply to the safety evaluation of biotechnology products with full recognition of the differences between the product types (Figure 20.6).

In cases where the target exists only in the disease state, animal models that mimic the human disease may be used to demonstrate binding to the target and pharmacological activity of the biotherapeutic. Indeed, a number of animal models have become available over the last 15 years for various disease states. It is not always clear, however, that the pharmacological activity in a rodent model will behave similarly to that in humans. When the pharmacological activity of a biopharmaceutical is dependent upon specific drug receptor/antigen binding that is not evident in an animal species, a number of different, scientifically rational approaches may be used to obtain these data, for example, xenograft models, transgenic models, etc. The NHP often is the animal model of choice in testing of these substances due to homology of the molecular targets in NHPs and humans; it is important to demonstrate the pharmacological relevance of the nonclinical species employed in safety assessment of a biologic.

Unlike traditional small molecules, biological drugs may have long half-lives *in vivo* (often humans \gg nonclinical species) and degradation usually occurs via nonenzymatic, proteolytic degradation processes. It is thus important that dose regimens in nonclinical species “cover” the pharmacokinetic and pharmacodynamic duration in humans. Once-a-week dosing and inclusion of recovery group animals that may be held for up to 3 months following treatment cessation (to ensure adequate drug clearance) may need to be considered. Of course, the issue of appropriate species needs to be considered, as noted earlier. A decrease in systemic half-life, even in animal models expressing the target of interest, often is the first sign that exposure to the biological drug has produced an antidrug immune response. Such findings may indicate the need for immunogenicity determinations either as part of subsequent nonclinical studies or in dedicated stand-alone studies to aid in interpretation of the animal studies. Such determinations are usually not relevant to assessment of immunogenicity risk in humans.

	Small molecule	Biologics
Molecular weight	Low	High
Manufacture	Chemical synthesis	Biosynthetic (cell-based)
Structure/purity of API	Pure single entity	Heterogeneous mixture
Usual route of administration	Oral, dermal, ocular, others	Intravenous, subcutaneous
Target distribution	Intra- and extra-cellular	Typically extra-cellular
Elimination	Metabolism (species differences)	Catabolism to amino acids
Toxicity	Off-target (parent or metabolite)	Exaggerated pharmacology
Species specificity	-/+	++++
Immunogenicity	-/+	++++

FIGURE 20.6 Biopharmaceuticals versus small molecules: Major differences that affect testing strategy.

FIH CLINICAL STUDIES

Initiation of the first human clinical study is the beginning of a long road to drug approval. In the absence of human experience, data from nonclinical toxicology and pharmacology studies are used to set safe starting doses in initial clinical trial. Although many details of determining the FIH trial designs are well beyond the scope of this chapter, there have been a number of reviews published on this topic.^{59–62}

Two approaches have been adopted for determining the FIH dose for most therapeutics (Figure 20.7). In the first approach, the NOAEL determined in pivotal nonclinical studies is identified in the most sensitive and relevant species.⁶³ The NOAEL is then adjusted with allometric scaling factors or on the basis of pharmacokinetic modeling to determine the human equivalent dose (HED). A safe starting dose in the clinic is estimated by applying an appropriate safety factor (typically 10) to the HED. The magnitude of the safety factor employed will clearly depend on the type of toxicity observed in the nonclinical studies, the dose response, and the design of the clinical trial (e.g., the use of healthy human volunteers versus patients) (Figure 20.8).

The conversion of the NOAEL to the HED is most often based on normalization to body surface area (BSA).⁶³ Conversion factors traditionally employed to calculate HED are shown in Table 20.8 and are derived from the following equation:

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times \left(\frac{W_A}{W_H} \right)^{0.33}$$

where

W_A and W_H are the weights of the experimental animal and human, respectively, in kilograms

W_H is usually assumed to be 60 kg

Example: If the NOAEL in most sensitive species (rat) is 10 mg/kg, the HED = 10 mg/kg \times 0.16 = 1.6 mg/kg.

In cases of potentially high risk drugs (novel mechanism of action, lack of relevant animal model, or dysregulation of immune function), a second, more conservative approach is employed to set initial human doses. The minimal anticipated biological effect level (MABEL) is a dose or concentration of a drug that results in a minimal biological (versus pharmacological or toxicological) effect to which appropriate safety factors can be applied to determine a safe FIH dose (Figure 20.9).⁶⁴

Oncological products represent a special case for determining the FIH dose.²⁹ For these products, it is not necessary to identify a NOAEL. The FIH dose for small molecules is set at 1/10th the severely toxic dose in 10% of the animals (STD10) for rodents or for nonrodents, 1/6th the highest non-severely toxic dose (HNSTD). The HNSTD is defined as the highest dose level that does not produce evidence of lethality, life-threatening toxicities, or irreversible findings.

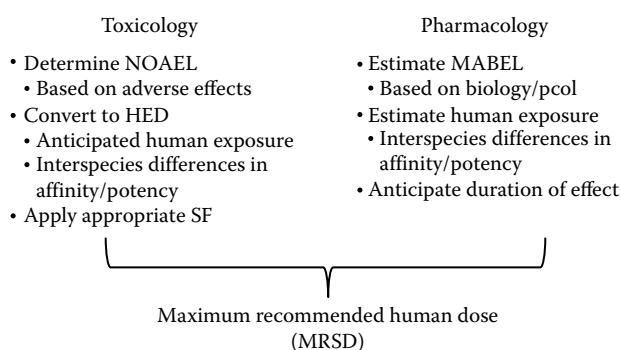


FIGURE 20.7 Approaches for determining MRSD for FIH clinical trials. HED, Human equivalent dose; MABEL, minimum anticipated biological effect level; MRSD, maximum recommended starting dose; NOAEL, no observable adverse effect level; SF, safety factor.

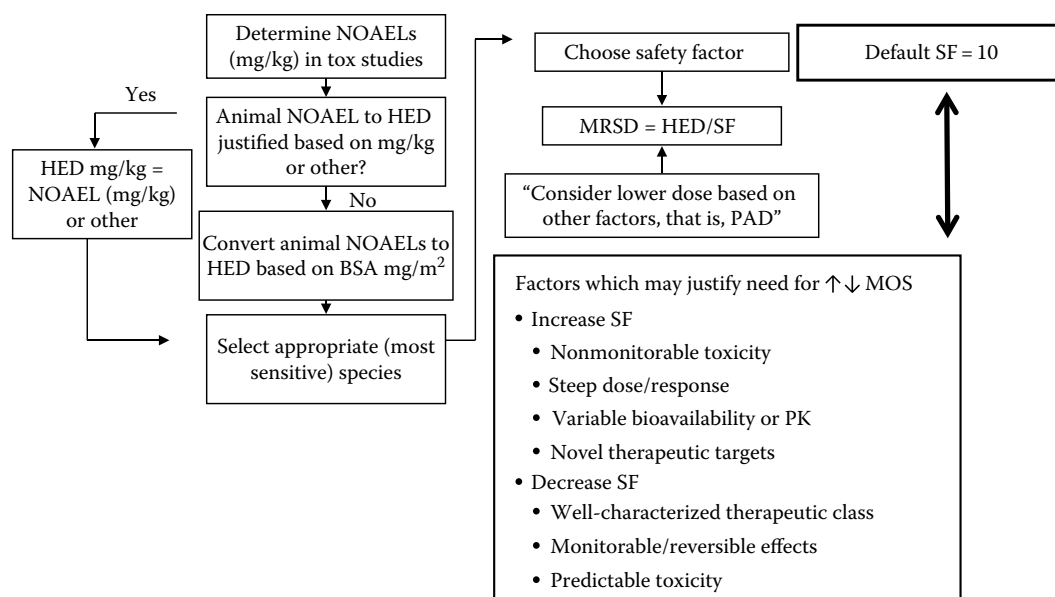


FIGURE 20.8 Clinical dose selection: Algorithm for estimating MRSD using the NOAEL HED, human equivalent dose; MOS, margin of safety; MRSD, maximum recommended starting dose; NOAEL, no observable adverse effect level; PK, pharmacokinetics; SF, safety factor.

TABLE 20.8

Conversion Factors of Animal Dose to Human Equivalent Dose (HED) Based on Body Surface Area for Selected Species

Species	To Convert Animal Dose (mg/kg) to Dose in mg/m ³ , Multiply by K_m	To Convert Animal Dose (mg/kg) to HED (mg/kg)	
		Divide Animal Dose by	Multiply Animal Dose by
Human			
Adult	37	—	—
Child	25	—	—
Mouse	3	12.3	0.08
Rat	6	6.2	0.16
Guinea pig	8	4.6	0.22
Rabbit	12	3.1	0.32
Dog	20	1.8	0.54
Primate			
Monkey	12	3.1	0.32
Marmoset	6	6.2	0.16
Baboon	20	1.8	0.54
Minipig	35	1.1	0.95

Source: Adapted from Food and Drug Administration (FDA), Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers, 2005, www.fda.gov.

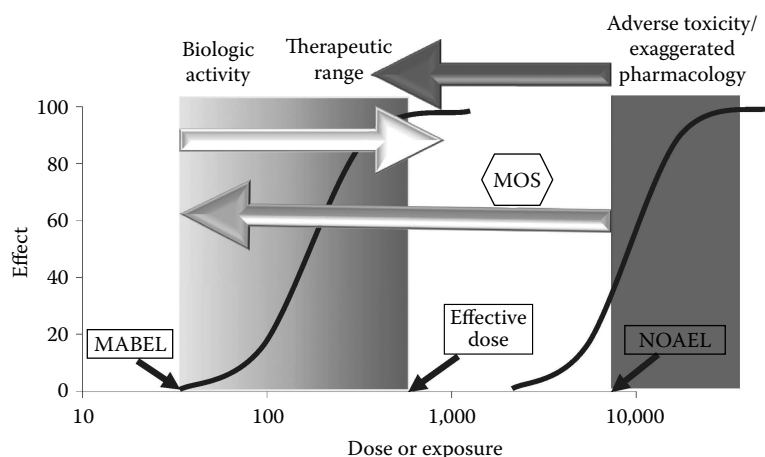


FIGURE 20.9 General overview of maximum recommended starting dose (MRSD). MABEL, Minimum anticipated biological effect level; MOS, margin of safety; NOAEL, no observable adverse effect level. (From Muller, P.Y., Mark Milton, M., Lloyd, P., Jennifer Sims, J., and Brennan, F.R., The minimum anticipated biological effect level [MABEL] for selection of first human dose in clinical trials with monoclonal antibodies, *Current Opinion in Biotechnology*, 20, 722, 2009.)

REFERENCES

1. IMS. 2012. IMS Forecasts & Reports. The Global Use of Medicines: Outlook through 2016. <http://www.imshealth.com/portal/site/ims/> (accessed July 2012).
2. DiMasi, J.A. 2001. Risks in new drug development. Approval success rates for investigational drugs. *Clin. Pharm. Therap.* 69: 297–307.
3. Pharmaceutical Research and Manufacturers of America (PhRMA). 2012. Profile Pharmaceutical Industry. www.phrma.org
4. Adams, C. and Brantner, V.V. 2010. Spending on new drug development. *Health Econ.* 19: 130–141.
5. Kaitin, K.I. and DiMasi, J.A. 2011. Pharmaceutical innovations in the 21st century: New drug approvals in the first decade, 2000–2009. *Clin. Pharmacol. Therap.* 89: 183–188.
6. Kola, I. and Landis, J. 2004. Can pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.* 3: 718.
7. Official Journal. 2006. Regulation (EC) No. 1901/2006 of the European Parliament and of the Council of 12 December 2006 on medicinal products for paediatric use and amending Regulation (EEC) No. 1768/92, directive 2001/20/EC, directive 2001/83/EC and Regulation (EC) No. 726/2004.
8. Food and Drug Administration (FDA). 1995. Guidance for Content and format of Investigational New Drug Applications (INDs) for Phase 1 studies of drugs, including well-characterized, therapeutic, biotechnology-derived products.
9. Mathieu, M. 2005. *New Drug Development: A Regulatory Overview*, 7th edition. Parexel, Waltham, MA.
10. International Conference on Harmonization (ICH). 2002. The common technical document (CTD).
11. International Conference on Harmonization (ICH). 2009. Guidance on the non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, M3(R2).
12. International Conference on Harmonization (ICH). 2006; 2011. Q3: Quality Guidelines; Impurities in drug substances, drug products, residual solvents.
13. Buckley, L.A. and Dorato, M.A. 2009. High dose selection in general toxicity studies for drug development: A pharmaceutical industry perspective. *Regul. Toxicol. Pharmacol.* 54: 301–307.
14. Schmued, L., Slikker, W., Clausing, P., and Bowyer, J. 1999. D-Fenfluramine produces neuronal degeneration in localized regions of the cortex, thalamus and cerebellum of the rat. *Toxicol. Sci.* 48: 100–106.
15. Schmued, L.C. and Hopkins, K.J. 2000. Fluoro-Jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res.* 874: 123–130.
16. Bolon, B., Garman, R., Jensen, K., Krinke, G., and Stuart, B. 2006. A “Best Practice” approach to neuropathic assessment in developmental neurotoxicity testing—For today. *Toxicol. Pathol.* 34: 296–313.
17. Lindgren, S., Bass, A.S., Briscoe, R., Bruce, K., Fredrichs, G.S., Kallman, M.J., Margaf, C., Patmore, L., and Pugsley, M.K. 2008. Benchmarking safety pharmacology regulatory packages and best practice. *J. Pharmacol. Toxicol. Methods.* 58: 99–109.
18. International Conference on Harmonization (ICH). 2011. Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, S2R1.
19. International Conference on Harmonization (ICH). 2001. International Conference on Harmonization: Safety pharmacology studies for human pharmaceuticals, S7.
20. Pugsley, M., Authier, S., and Curtis, M.J. 2008. Principles of safety pharmacology. *Br. J. Pharmacol.* 154: 1382–1399.
21. Authier, S., Gervais, J., Fournier, S., Gauvin, D., Maghezzi, S., and Troncy, S. 2011. Cardiovascular and respiratory safety pharmacology in Gottingen minipigs: Pharmacological characterization. *J. Pharmacol. Toxicol. Methods.* 64: 53–59.
22. Authier, S., Legaspi, M., Gauvin, D., and Troncy, E. 2009. Respiratory safety pharmacology: Positive control drug responses in Sprague-Dawley rats, beagle dogs and cynomolgus monkeys. *Regul. Toxicol. Pharmacol.* 55: 229–235.
23. Authier, S., Legaspi, M., Gauvin, D., Chaurand, F., Fournier, S., and Troncy, E. 2008. Validation of respiratory safety pharmacology models: Conscious and anesthetized beagle dogs. *J. Pharmacol. Toxicol. Methods.* 57: 52–60.

24. Gauvin, D.V., Tilley, L.P., Smith, F.W.K., and Baird, T.J. 2006. Electrocardiogram, hemodynamics, and core body temperature of the freely moving laboratory beagle dog by remote telemetry. *J. Pharmacol. Toxicol. Methods*. 53: 128–139.
25. Cheeseman, M.A., Machuga, E.J., and Bailey, A.B. 1999. A tiered approach to threshold of regulation. *Food Chem. Toxicol.* 37: 387–412.
26. Kirkland, D., Aardema, M., Henderson, L., and Muller, L. 2005. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity, and relative predictivity. *Mutat. Res.* 584: 1–256.
27. International Conference on Harmonization (ICH). 2005. Detection of toxicity to reproduction for medicinal products and toxicity to male fertility, S5R2.
28. International Conference on Harmonization (ICH). 1997. Need for carcinogenicity studies of pharmaceuticals, S1A.
29. International Conference on Harmonization (ICH). 2009. S9: Nonclinical evaluation for anticancer pharmaceuticals.
30. DeGeorge, J., Ahn, C., Andrews, P., Bower, M., Giorgio, D., Goheer, M., Lee-Yam, D., McGuinn, W., Schmidt, W., Sun, C., and Tripathi, S. 1998. Regulatory considerations for the preclinical development of anticancer drugs. *Cancer Chemother. Pharmacol.* 41: 173–185.
31. International Conference on Harmonization (ICH). 1997. Testing for carcinogenicity of pharmaceuticals, S1B.
32. International Conference on Harmonization (ICH). 2008. Dose selection for carcinogenicity studies of pharmaceuticals, S1C(R2).
33. Rhomberg, R.L., Baetche, K., Blancato, J., Bus, J., Cohen, S., Conolly, R., Dixit, R. et al. 2007. Issues in the design and interpretation of chronic toxicity and carcinogenicity studies: Approaches to dose selection. *Crit. Rev. Toxicol.* 37: 729–837.
34. Reddy, V.M., Sistare, F.D., Christensen, J.S., DeLuca, J.G., Wollenberg, G.K., and DeGeorge, J.J. 2010. An evaluation of chronic 6- and 12-month rat toxicology studies as predictors of 2-year tumor outcome. *Vet. Pathol.* 47: 614–629.
35. Pritchard, J.B., French, J.E., Davis, B.J., and Haseman, J.K. 2003. The role of transgenic mouse models in carcinogen identification. *Environ. Health Persp.* 111: 444–454.
36. Food and Drug Administration (FDA). 2002. Guidance for industry: Carcinogenicity study protocol submissions, FDA, Rockville, MD.
37. Young, J.K., Hall, R.L., O'Brien, P., Strauss, V., and Vahle, J.L. 2011. Best practices for clinical pathology testing in carcinogenicity studies. *Toxicol. Pathol.* 39: 429–434.
38. Vahle, J.L., Finch, G.L., Heidel, S.M., Hovland, D.N., Ivens, I., Parker, S., Ponce, R.A., Sachs, C., Steigerwalt, R., Short, B., and Todd, M. 2010. Carcinogenicity assessment of biotechnology-derived pharmaceuticals: A review of approved molecules and best practices recommendations. *Toxicol. Pathol.* 38: 522–553.
39. Food and Drug Administration (FDA). 2006. Guidance for industry: Nonclinical safety evaluation of pediatric drug products, FDA, Rockville, MD.
40. European Medicines Evaluation Agency (EMA), Committee for Human Medicinal Products (CHMP). 2008. Guideline on the need for non-clinical testing in juvenile animals on human pharmaceuticals for pediatric indications.
41. Hurtt, M.E., Daston, G., Davis-Bruno, K., Feuston, M., Silva-Lima, B., Makris, S., Mc Nerney, M.E., Sandler, J.D., Whitby, K., Wier, P., and Cappon, G.D. 2004. Juvenile animal studies: Testing strategy and design. *Birth Defects Res. (Pt B)*. 71: 281–288.
42. Cappon, G., Bailey, G., Buschmann, J., Feuston, M.H., Fisher, J.E., Hew, K.W., Hoberman, A.M., Ooshima, Y., Stump, D.G., and Hurtt, M.E. 2009. Juvenile animal toxicity study designs to support pediatric drug development. *Birth Defects Res. (Pt B)*. 86: 463–469.
43. Baldrick, P. 2004. Developing drugs for paediatric use: A role for juvenile animal studies? *Reg. Toxicol. Pharmacol.* 39: 381–389.
44. Anderson, T., Khan, N.K., Tassinari, M.S., and Hurtt, M.E. 2009. Comparative juvenile safety testing of new therapeutic candidates: Relevance of laboratory animal data to children. *J. Toxicol. Sci.* 34(Special Issue II): SP209–SP215.
45. Beck, M.J., Padgett, E.L., Bowman, C.J., Wilson, D.T., Kaufman, L.E., Varsho, B.J., Stump, D.G., Nemec, M.D., and Holson, J.F. 2006. Nonclinical juvenile toxicity testing. In: *Developmental and Reproductive Toxicology. A Practical Approach*, Hood, R.D., ed. CRC Press, Taylor & Francis Group, Boca Raton, FL.
46. De Schaepdrijver, L.M. and Bailey, G.P. 2009. Preclinical juvenile toxicity assessments and study designs. In: *Pediatric Drug Development: Concepts and Applications*. Mulberg, A.E., Silber, S.S., and van den Anker, J.N., eds. John Wiley & Sons, Hoboken, NJ.
47. Buelke-Sam, J. 2003. Comparative schedules of development in rats and humans: Implications for developmental neurotoxicity testing. *Presented at the Annual Meeting of the Society of Toxicology*, Salt Lake City, UT.
48. Hew, K.W. and Keller, K.A. 2003. Postnatal anatomical and functional development of the heart: A species comparison. *Birth Defects Res. (Pt B)*. 68: 309–320.
49. Zoetis, T. and Hurtt, M.E. 2003. Species comparison of lung development. *Birth Defects Res. (Pt B)*. 68: 121–124.
50. Zoetis, T. and Hurtt, M.E. 2003. Species comparison of anatomical and functional renal development. *Birth Defects Res. (Pt B)*. 68: 111–120.
51. Watson, R.E., DeSesso, J.M., Hurtt, M.E., and Cappon, G.D. 2006. Postnatal growth and morphological development of the brain: A species comparison. *Birth Defects Res. (Pt B)*. 77: 471–484.
52. Wood, S.L., Beyer, B.K., and Cappon, G.D. 2003. Species comparison of postnatal CNS development: Functional measures. *Birth Defects Res. (Pt B)*. 68: 391–407.
53. Holsapple, M.P., West, L.J., and Landreth, K.S. 2003. Species comparison of anatomical and functional immune system development. *Birth Defects Res. (Pt B)*. 68: 321–334.
54. Marty, M.S., Chapin, R.E., Parks, L.G., and Thorsrud, B.A. 2003. Development and maturation of the male reproductive system. *Birth Defects Res. (Pt B)*. 68: 125–136.
55. Beckman, D.A. and Feuston, M. 2003. Landmarks in the development of the female reproductive system. *Birth Defects Res. (Part B)*. 68: 137–143.
56. Walthall, K., Cappon, G.D., Hurtt, M.E., and Zoetis, T. 2004. Postnatal development of the gastrointestinal system: A species comparison. *Birth Defects Res. (Pt B)*. 74: 132–156.

57. Zoetis, T., Tassinari, M.S., Bagi, C., Walthall, K., and Hurtt, M.E. 2003. Species comparison of postnatal bone growth and development. *Birth Defects Res. (Pt B)*. 68: 86–110.
58. International Conference on Harmonization (ICH). 2011b. Preclinical safety evaluation of biotechnology-derived pharmaceuticals, S6R1.
59. Tibbitts, J., Cavagnaro, J.A., Haller, C.A., Marafino, B., Andrews, P.A., and Sullivan, J.T. 2010. Practical approaches to dose selection for first-in-human clinical trials with novel biopharmaceuticals. *Reg. Toxicol. Pharmacol.* 58: 243–251.
60. Sharma, V. and McNeill, J.H. 2009. To scale or not to scale: The principles of dose extrapolation. *Br. J. Pharmacol.* 157: 907–921.
61. Mager, D.E., Woo, S., and Jusko, W.J. 2009. Scaling pharmacodynamics from *in vitro* and preclinical animal studies to humans. *Drug Metab. Pharmacokinet.* 24: 16–24.
62. Buckley, L.A., Garhyan, P., Ponce, R., and Roberts, S.A. 2010. Estimation of human starting dose for Phase 1 clinical programs, Chapter 10. In: *Early Drug Development: Strategies and Routes to First-in-Human Trials*, Cayen, M.N., ed. Wiley & Sons, NJ, pp. 423–464.
63. Food and Drug Administration (FDA). 2005. Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. www.fda.gov.
64. Committee for Medicinal Products for Human Use (CHMP). 2007. Guideline on requirements for first-in-man clinical trials for potential high-risk medicinal products.
65. IMS. 2012. Top-Line Market Data. Top 20 Global Products, 2011. <http://www.imshealth.com/portal/site/ims/> (accessed on July, 2012).
66. Dorato, M.A., McMillian, C.L., and Vodcnik, M.J. 2008. The toxicologic assessment of pharmaceutical and biotechnology products. In: *Principles and Methods of Toxicology*, 5th edn., A.W. Hayes (ed.). CRC Press, New York, pp. 325–368.
67. Muller, P.Y., Mark Milton, M., Lloyd, P., Jennifer Sims, J., and Brennan, F.R. 2009. The minimum anticipated biological effect level (MABEL) for selection of first human dose in clinical trials with monoclonal antibodies. *Current Opinion in Biotechnology*, 20:722–729.

Website Links:

- European Medicines Agency (EMA). www.emea.eu.int
- Food and Drug Administration, Center for Drug Evaluation and Research. www.fda.gov/cder.
- Food and Drug Administration, Center for Biologics Evaluation and Research. www.fda.gov/BiologicsBloodVaccines/default.htm.
- International Conference on Harmonization. www.ich.org.
- Japan Pharmaceutical Manufacturer Association (JPMA). www.jpma.or.jp/english.
- Organization for Economic Cooperation and Development (OECD). www.oecd.org.
- Pharmaceutical Research and Manufacturers of America (PhRMA). <http://www.phrma.org/>.

21 Safety Pharmacology

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INTRODUCTION

Safety pharmacology is a regulatory science grounded in physiology, pharmacology, and toxicology, the primary goal of which is to protect human health from potentially adverse, off-target drug activity, particularly in early clinical trials, but also throughout the development and marketing cycle. It is rooted in the early recognition that the acute functional effects of drugs on physiological systems represent a potential hazard that is at least as important as classic toxicological interactions in terms of impact on health risk and the potential for compound failure in the drug development process. It is increasingly understood that these off-target functional effects of interest are also those which elude appropriate characterization in safety programs excluding the incorporation of specific function tests on systems critical to the (often short-term) maintenance of homeostasis. The research models, technology, and environment required for ideal interrogation of such systems are often outside the scope of traditional toxicological evaluation, and accordingly must be addressed by alternative means. The main goals of this chapter are to distinguish safety pharmacology from related endeavors, such as traditional drug toxicity testing, provide translational information tying relevant regulatory guidance to practical contemporary safety pharmacology designs, discuss factors intrinsic to sound safety pharmacology study construct, and provide relevant historical control data for core assessments with a sampling of relevant species and technologies. Additionally, a goal of this chapter is to demonstrate how different types of molecules guide the approach applied to

functional safety evaluation, and illustrate circumstances in which stand-alone or integrated safety pharmacology programs are utilized to add value to programmatic efforts in drug safety evaluation.

SAFETY PHARMACOLOGY HARMONIZED GUIDELINES

Among the earliest regulatory references to experimental paradigms consistent with that which we understand today as safety pharmacology investigations are those contained within Japanese regulatory history.¹⁻³ This history, with contribution from other established regional regulatory authorities, has contributed significantly to the development of internationally recognized harmonized guidelines, ICH S7A, “Safety Pharmacology Studies for Human Pharmaceuticals,”⁴ and ICH S7B “Guidance on Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals.”⁵ Based on expert input, these guidance documents have articulated formal recommendations for design, execution, and interpretive elements of safety pharmacology investigations in core and ancillary functions,^{6,7} including a discussion of techniques considered appropriate for determining the potential for a drug to produce alterations in patterns of cardiac conduction, especially as it relates to ventricular repolarization status. The development and promulgation of such internationally harmonized guidance documents

has occurred in the context of a history of difficult circumstances surrounding unrecognized (cardiovascular) risks accompanying industry practice prior to their inception.^{8–12} While the issuance of regulatory guidance has not resulted in, nor reasonably may be expected to lead to the complete mitigation of risk, recommendations within these guidance documents have been put forth with the goal of enhancing, to the extent possible, the reliability and predictive validity of preclinical models used for the purpose of characterizing functional safety concerns. While specific in some respects (e.g., recommendations on GLP status of different kinds of safety pharmacology studies, essential end points for characterization in core study designs), these guidance documents are notably, and it might be argued, necessarily more generic in the other sections relating to the specifics of model selection and study design (e.g., species of choice, numbers of subjects and test conditions, etc.). This circumstance has been acknowledged in terms of facilitating an understanding of the goals of testing without unduly dictating application of a particular approach without appropriate consideration of the unique circumstances intrinsic to each development program. Accordingly, in the sections to follow, it should be realized that presentation of a “typical” study design or evaluative technique is done so only with the goal of serving as a point of reference for instructional purposes, and is not intended to be applicable to every situation.

SAMPLE STUDY DESIGNS

CARDIOVASCULAR FUNCTION STUDIES

The cardiovascular system is complex, with specialized tissues and coordinated functions that support multiple vital homeostatic mechanisms. A comprehensive overview of cardiovascular anatomy and physiology, and the numerous models applicable to cardiovascular safety assessment is beyond the scope of this chapter; however, relevant information may be found in various sources.^{13–15} The cardiovascular system may be examined at many different levels and utilizing diverse methods to evaluate aspects of drug action, at the level of subcellular components, to collections of tissues performing coordinated functions. While many assays are of potential value regarding the characterization of drug action on the cardiovascular system, contemporary regulatory authority, and correspondingly contemporary practice, has focused on a relatively limited set of key indicators to be employed at a minimum prior to administration of a new chemical entity in humans. The essential first tier tests include assessment of the potential for cardiac ion channel interactions, and evaluation of several global measures of cardiovascular function, including arterial blood pressure, heart rate, qualitative and quantitative electrophysiological features^{4,5} (see Table 21.1).

The text tables represent variants on some common *in vivo* cardiovascular safety pharmacology study designs in rodents and nonrodents which are consistent with

current interpretations of regulatory guidance and industry best practices.^{16–20} As in the subsequent sections, the study designs presented are accompanied by explanatory footnotes which clarify important scientific and regulatory rationales influencing key experimental design characteristics. *In vitro* evaluations, such as are suitable for characterizing a range of activity, from ion channel interactions, to coordinated electromechanical conduction in *ex vivo* tissues, are available from a variety of sources, including historical and positive control data, and so will not be presented in detail herein.^{21–26} Core cardiovascular assessments for regulatory submission (Good Laboratory Practices: GLP-compliant) are generally conducted in accepted large animal test systems, while early screening for selected end points or directed investigations into specific effects and/or mechanisms of action may be conducted more routinely in other (rodent, guinea pig, rabbit) models, often on a non-GLP basis. Since the ICH S7A and ICH S7B guidance documents recommend use of telemetry for *in vivo* cardiovascular studies to avoid potential disruptive or confounding influences of restraint and/or anesthesia on sensitive physiological measures, there is a corresponding focus on designs utilizing this technology. However, it should be understood that anesthetized cardiovascular preparations in both large and small animal test systems have a vital role in contributing to understandings of potential cardiovascular liabilities, allowing access additionally to a variety of supplemental parameters that are not routinely accessible by currently employed telemetric techniques.²⁷ Accordingly, the utilization of anesthetized models with an expanded array of cardiovascular indicator variables fills a critical role in early-phase study designs utilized to characterize a wide range of potential safety issues, as well as directed studies intended to further investigate mechanisms of action and potential reversibility of drug effects of concern on the cardiovascular system. In addition, such anesthetized designs are useful when the feasibility of dosing up to an intended range is limited by factors such as emesis and lack of tolerability, in addition to other practical considerations. In comparison to large animals, small animal test systems are generally equally amenable to evaluation by means of anesthetized cardiovascular techniques, with some notable exceptions related to predictive validity of experimental outcome (e.g., assessment of cardiac repolarization, as described in the table under “Experimental Design—Cardiovascular Safety Evaluation by Telemetry in Rodents or Guinea Pigs”). Since a sample study design, as performed in small animals accordingly, may be considered largely redundant to that conducted in large animals, depiction of a general study outline in this section is limited to that indicated for large animal species.

An important aspect of conducting core battery safety pharmacology, including cardiovascular function studies, is developing appropriate historical control data to understand essential assay variability associated with contributing elements unique to independent laboratories, such as

test system, technology employed, environmental and procedural variables, statistical analysis routines, and other factors.^{19,20,26} Standard descriptive statistical measures of central tendency and variability are often instructive at a basic level regarding assay stability.^{28,29} However, specific evaluations of statistical power may also be performed on baseline cardiovascular function variables to yield a more formal analysis of assay sensitivity.³⁰ Of course, application of appropriate positive control articles, ideally effecting bidirectional change on key indicator variables, is a common and established approach to demonstrating assay sensitivity and selectivity.^{31,32} Such active comparator evaluations need not be incorporated within the context of every individual study performed, but may be conducted on a periodic basis within individual testing facilities to allow ongoing monitoring of assay performance over time. Statistical evaluations on individual studies, if performed, may range from simple descriptive statistics to Analysis of Variance (ANOVA) or Repeated Measures Analysis of Covariance (RMANCOVA) procedures, depending upon individual institutional practices. Ultimately, statistical modeling may be most appropriately considered an aid for decision making in situations where clear dose–response pharmacology is not in evidence, and is not strictly required where biologically and/or pharmacologically meaningful, dose–response elicitation of parameter change has been induced by appropriate manipulation of independent variables with corresponding controls on inherent assay variability. Beyond inferential statistical modeling, often assessment of pharmacokinetic/pharmacodynamic (PK/PD) relationships is instructive in gaining a full appreciation of the test system and model characteristics and allowing some level of prediction to facilitate planning for early clinical studies that rely upon preclinical data for clarifying and managing risk.

TABLE 21.1
Examples of Core Battery (Tier I) and Supplemental (Tier II) Cardiovascular Function Measures Investigated in Safety Pharmacology Studies

Cardiovascular Safety Pharmacology Measure	Core Battery (Tier I)	Supplemental (Tier II)
hERG interaction	X	
Heart rate and rhythm	X	
Blood pressure	X	
Quantitative ECG parameters (PR, QRS, QT, QTc)	X	
Ventricular pressure and derivatives (e.g., $\pm dPdt$)		X
Vascular compliance/resistance		X
Cardiac output		X

TABLE 21.1 (continued)
Examples of Core Battery (Tier I) and Supplemental (Tier II) Cardiovascular Function Measures Investigated in Safety Pharmacology Studies

Cardiovascular Safety Pharmacology Measure	Core Battery (Tier I)	Supplemental (Tier II)
Estimates of myocardial work (e.g., CI, SI, LVW)		X
Biomarkers for cardiotoxicity (e.g., CK-MB isoforms, cTnI, BNP, CRP, IL-6, TNF- α)		X

EXPERIMENTAL DESIGN— CARDIOVASCULAR SAFETY EVALUATION BY TELEMETRY IN NONRODENTS

Test system: 4–8 experimentally naïve or nonnaïve animals per treatment/group*

Study design: The study is most commonly conducted according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route in animals that are instrumented with calibrated telemetric devices and data are collected using associated software control systems capable of detecting and/or deriving a variety of cardiovascular parameters via raw pressure and biopotential signals. Either independent group or within-subjects designs may be utilized, although within-subjects designs are the most common approach. If conducted using a within-subjects design, dose administration typically proceeds according to a Latin square or ascending dose sequence, with an appropriate recovery period between treatments based on known PK/PD profiles. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound and metabolites, as applicable. Telemetry device implantation

* Experimentally naïve or nonnaïve animals are considered acceptable. If nonnaïve (e.g., often telemetry stock colony) animals are utilized, health status qualifications (clinical pathology, physical examinations, clinical observations, ECGs) are performed to permit assignment of individual animals to study. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The canine (beagle) and nonhuman primate (cynomolgus or rhesus monkey) are well-accepted species for which a large historical database is available; lesser utilized models include swine (typically minipigs), marmosets, and ferrets.

requires surgery and typically 10–14 days recovery prior to the initiation of data collection and dosing. Acclimation to the laboratory environment requires transfer of the animals to the study room at least 1 week prior to study initiation.

Treatment/Condition	Number of Animals
Baseline (24 h) ^a	4–8
Negative (vehicle) control	4–8
Active Drug Dose 1	4–8
Active Drug Dose 2	4–8
Active Drug Dose 3	4–8

^a An ~24 h period of baseline data are collected prior to dosing initiation to allow evaluation of telemetry signal quality, confirmation of animal suitability, and provision of an appropriate data record permitting establishment of individual animal QT/RR relationships for probabilistic QT interval correction.

Dependent measures:

- Systolic, diastolic, and mean arterial blood pressures
- Heart rate (derived from electrocardiogram [ECG] or blood pressure signal)
- ECG: Quantitative intervals (RR, PR, QRS, QT, and QTc), qualitative assessment*
- Ventricular pressure and derivatives (estimates of contractility, relaxation, etc.—optional)[†]
- Body temperature, general activity (optional)[‡]
- Clinical observations
- Body weights

* Representative ECG tracings of appropriate duration (defined institutionally according to the goals of evaluation) are collected prior to exposure, at appropriate interval(s) postdose based on drug PK/PD characteristics, and near the end of the cardiovascular monitoring period for each treatment. Evaluation over these intervals permits an assessment of baseline, peak effect, and duration of action. ECG records are reviewed by a board-certified veterinary cardiologist, or otherwise qualified expert.

[†] These parameters are accessible using standard available telemetry devices, and are particularly relevant in circumstances where a drug class effect may be of concern.

[‡] Core body temperature requires appropriate placement of the temperature sensing component of the telemetry device; subcutaneous location of the device does not permit accurate recordings of core body temperature. Core temperature data are useful as a general indicator of potential CNS activity (as is general activity), as well as in circumstances where thermoregulation is affected (e.g., hypothermia) to the extent that correction of QTc values for body temperature is warranted.

EXPERIMENTAL DESIGN—ANESTHETIZED/RESTRAINED CARDIOVASCULAR SAFETY EVALUATION IN NONRODENTS

Test system: 3–6 experimentally naïve or nonnaïve animals per treatment/group*

Study design: The study may be conducted non-GLP, or according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route (often intravenous) in animals that are acutely instrumented with appropriately calibrated monitoring devices, and data are collected using associated software control systems capable of detecting and/or deriving a variety of cardiovascular parameters via raw pressure and biopotential signals. Either independent group or within-subjects designs may be utilized, although within-subjects designs are the most common approach, often with ascending/cumulative dosing regimens performed over several hours while animals are physically or chemically restrained. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound; due to the limited duration of recording, often up to a maximum of just several hours, evaluation for metabolite effects may be more difficult in this context. Once animals are appropriately instrumented, a stable baseline facilitated by either level surgical plane of anesthesia or appropriate acclimation to the restraint condition is initially collected, followed by experimental treatments. Acclimation to the laboratory conditions when using a method of physical restraint often requires several days of procedural habituation prior to study initiation.

* Experimentally naïve or nonnaïve animals are considered acceptable, although nonnaïve animal utilization is somewhat less relevant to anesthetized preparations, as these often are terminal procedures. If nonnaïve animals are utilized, health status qualifications (clinical pathology, physical examinations, clinical observations, ECGs) are performed to permit assignment of individual animals to study. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The canine (beagle) is a well-accepted species for which a large historical database is available; lesser utilized models include nonhuman primates (cynomolgus or rhesus monkeys), swine (typically minipigs), rabbits, marmosets, and ferrets. Due to an enhanced ability to control environmental variables and the maximum range of drug exposures in anesthetized models (e.g., enhanced internal validity), fewer animals may be utilized in relation to designs employing unrestrained telemetric techniques in order to draw clear and valid conclusions.

Treatment/Condition	Number of Animals
Baseline ^a	3–6
Negative (vehicle) control	3–6
Active Drug Dose 1	3–6
Active Drug Dose 2	3–6
Active Drug Dose 3	3–6

^a An appropriate duration of baseline data, often at least 0.5–1 h, are collected immediately prior to each treatment condition. This data collection period may be utilized to positively establish signal quality and confirm animal/preparation suitability.

Dependent measures:

- Systolic, diastolic, and mean arterial blood pressures
- Heart rate (derived from ECG or blood pressure signal)
- ECG: Quantitative intervals (RR, PR, QRS, QT, and QTc), qualitative assessment*
- Ventricular pressure and derivatives (estimates of contractility, relaxation, etc.)[†]
- Vascular compliance/resistance[†]
- Cardiac output[†]
- Estimates of myocardial work[†]
- Body temperature (optional)[‡]
- Clinical observations (generally more applicable to nonanesthetized preparations)
- Body weights

* Representative ECG tracings of appropriate duration (defined institutionally according to the goals of evaluation) are collected prior to exposure, at appropriate interval(s) postdose based on drug PK/PD characteristics, and near the end of the cardiovascular monitoring period for each treatment. Evaluation over these intervals permits an assessment of baseline, peak effect, and duration of action. ECG records are reviewed by a board-certified veterinary cardiologist, or otherwise qualified expert.

[†] These and other parameters are particularly relevant in circumstances where a drug class effect may be of concern, and are generally more accessible in anesthetized models.

[‡] Core body temperature data are useful as a general indicator of potential CNS activity, as well as in circumstances where thermoregulation is affected (e.g., hypothermia) to the extent that correction of QTc values for body temperature is warranted. During anesthetized procedures, core temperature should be monitored along with plane of surgical anesthesia in order to keep these regulated within a stable range; doing so will preempt the need for QTc correction for body temperature changes, and may also help to mitigate heart rate variability. In cases where active drug treatments affect body temperature, or core temperature otherwise varies in an uncontrolled manner, having a sufficient number of temporally correlated QT/QTc and core body temperature values will allow QTc correction for such body temperature variations. Alternatively, correction factors have been proposed for various species and may be found in the scientific literature.

EXPERIMENTAL DESIGN— CARDIOVASCULAR SAFETY EVALUATION BY TELEMETRY IN RODENTS OR GUINEA PIGS

Test system: 4–8 experimentally naïve or nonnaïve animals per treatment/group*

Study design: The study may be conducted non-GLP, or according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route in animals that are instrumented with calibrated telemetric devices and data are collected using associated software control systems capable of detecting and/or deriving a variety of cardiovascular parameters via raw pressure and/or biopotential signals. Either independent group or within-subjects designs may be utilized. If conducted using a within-subjects design, dose administration typically proceeds according to a Latin square or ascending dose sequence, with an appropriate recovery period between treatments based on known PK/PD profiles. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound and metabolites, as applicable. Telemetry device implantation requires surgery and typically 7–14 days recovery prior to the initiation of data collection and dosing. Acclimation to the laboratory environment requires transfer of the animals to the study room at least 1 week prior to study initiation.

Treatment/Condition	Number of Animals
Baseline (24 h) ^a	4–8
Negative (vehicle) control	4–8
Active Drug Dose 1	4–8
Active Drug Dose 2	4–8
Active Drug Dose 3	4–8

^a A suitable period of baseline data may be collected prior to dosing initiation to allow evaluation of telemetry signal quality and confirmation of animal suitability.

* Experimentally naïve or nonnaïve animals are considered acceptable. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The mouse and rat represent appropriate models for blood pressure assessments in general. However, due to high resting heart rates and ion channels mediating repolarization, these species are not generally accepted models for definitive characterization of conductance and repolarization abnormalities (e.g., ICH S7B).

Dependent measures:

- Systolic, diastolic, and mean arterial blood pressures
- Heart rate (derived from ECG or blood pressure signal)
- ECG: Quantitative intervals (RR, PR, QRS, QT, and QTc), qualitative assessment*
- Estimates of ventricular contractility and relaxation†
- Body temperature, general activity‡
- Clinical observations
- Body weights

* Collection of ECG data in guinea pigs for the purpose of evaluation for conductance and repolarization abnormalities is acceptable according to ICH S7B and contemporary scientific standards; mice and rats are not considered acceptable for modeling these effects.

† These parameters are accessible using standard available telemetry devices, and are particularly relevant in circumstances where a drug class effect may be of concern.

‡ Core body temperature requires appropriate placement of the temperature sensing component of the telemetry device; subcutaneous location of the device does not permit accurate recordings of core body temperature. Core temperature (as well as general activity) data are useful as a general indicator of potential CNS activity; temperature data sampling is also relevant in circumstances where thermoregulation is affected (e.g., hypothermia) to the extent that correction of QTc values for body temperature is warranted. Core temperature, by appropriate placement of the telemetry device within the abdominal cavity, is accessible in the guinea pig, but may be somewhat less so in the rat and mouse, depending on surgical approach and specific telemetry device employed.

RESPIRATORY FUNCTION STUDIES

Assessment of the potential for off-target and potentially adverse drug effects on respiratory functions may involve investigations of the mechanical conduction system comprised of conducting airways and neuromuscular control of the ventilatory apparatus, as well as of the lung mechanics (resistance/compliance) and gas exchange functions mediated by deeper alveolar–vascular networks.³³ While a comprehensive review of respiratory system anatomy and physiology is beyond the scope of the present discussion, such information is available from various sources.^{13,34,35} To meet essential standards for Tier I safety evaluation, contemporary practice involves at minimum an assessment of respiratory rate and volume, or some combination of rate and another index of functional gas exchange, such as may be yielded by blood gas analyses (Table 21.2).

According to the same organizational format of the preceding section on cardiovascular function assessment, the following tables represent variants on some

common respiratory safety pharmacology study designs in rodents and nonrodents which are consistent with current interpretations of regulatory guidance and industry best practices.^{4,5,16–18,26} Core respiratory assessments for regulatory submission (GLP) are generally conducted in accepted small or large animal test systems, while early screening for selected end points or directed investigations may be conducted more routinely in rodent models, often on a non-GLP basis. Although the ICH S7A guidance document recommends use of unrestrained techniques for *in vivo* respiratory function assessment to avoid potential disruptive or confounding influences of restraint and/or anesthesia, designs utilizing both unrestrained and restrained techniques are presented for consideration, and these alternative methods are considered appropriate under varying circumstances. As is the case with cardiovascular function testing, it should be understood that anesthetized preparations play a vital role in contributing to the appreciation of some potential respiratory function liabilities, including end points that are not routinely accessible by currently employed unrestrained and nonanesthetized techniques.^{13,33–36} Accordingly, the utilization of anesthetized models including an expanded array of respiratory mechanics, tissue resistance and compliance, and gas exchange functional characteristics fills a critical role in directed studies designed to further investigate mechanisms of action and potential reversibility of drug effects of concern on the respiratory system. In addition, and also consistent with cardiovascular function testing, such anesthetized designs are useful when the feasibility of dosing up to an intended range is limited by factors such as emesis and lack of tolerability, in addition to other practical considerations. Also, because of the continuous, time-series nature of data produced by standard study designs, statistical evaluations, if performed, accordingly may range from simple descriptive techniques to ANOVA or RMANCOVA procedures, depending upon individual institutional practices. Particularly in the case of some of the essential respiratory mechanics parameters (volume, rate), there exists a wide range of biologically normal variability, as demonstrated by nonpharmacological, circadian, or other environmental stimuli (handling, dosing procedures, etc.)—associated fluctuations in these measures. The range of variability of changes associated with such stimuli may routinely far exceed that identified as a “statistically significant” difference detected by a given testing routine. Accordingly, and consistent with the aims of safety pharmacology studies in general, statistical evaluations may be best utilized as a guide toward facilitating interpretation in situations where clear dose–response pharmacology is not in evidence, and is not strictly required where biologically and/or pharmacologically meaningful, dose–response elicitation of parameter change has been induced by appropriate manipulation of independent variables with appropriate corresponding controls on inherent assay variability.

TABLE 21.2
Examples of Core Battery (Tier I) and Supplemental (Tier II) Respiratory Function Measures Investigated in Safety Pharmacology Studies

Respiratory Safety Pharmacology Measure	Core Battery (Tier I)	Supplemental (Tier II)
Respiratory rate	X	
Tidal volume	X	
Minute volume	X	
Resistance/compliance		X
Pulmonary hemodynamics		X
Gas exchange (blood gas, pH, co-oximetry)		X

EXPERIMENTAL DESIGN— RESPIRATORY SAFETY EVALUATION IN CONSCIOUS NONRODENTS

Test system: 4–8 experimentally naïve or nonnaïve animals per treatment/group*

Study design: The study is most commonly conducted according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route in animals that are sling (minipig, canine) or

* Experimentally naïve or nonnaïve animals are considered acceptable. If nonnaïve animals are utilized, particularly animals derived from telemetry stock colonies, health status qualifications (clinical pathology, physical examinations, clinical observations, ECGs) may be performed to permit assignment of individual animals to study. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The canine (beagle) and nonhuman primate (cynomolgus or rhesus monkey) are well-accepted species for which a large historical database is available; lesser utilized models include swine (typically minipigs) and marmosets.

chair (nonhuman primate) restrained and fitted with a head plethysmograph or nose-cone/mask articulated to a computerized data collection system capable of detecting and/or deriving a variety of essential parameters via raw pressure signals. Either independent group or within-subjects designs may be utilized, although within-subjects designs are the most common approach. If conducted using a within-subjects design, dose administration typically proceeds according to a Latin square or ascending dose sequence, with an appropriate recovery period between treatments based on known PK/PD profiles. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound; due to the limited duration of recording, often up to a maximum of just several hours, evaluation for metabolite effects may be more difficult in this context. Once animals are appropriately configured, a stable baseline facilitated by acclimation to the restraint condition is initially collected, followed by experimental treatments. Acclimation to the laboratory conditions when using a method of physical restraint often requires several days of procedural habituation prior to study initiation.

Treatment/Condition	Number of Animals
Baseline ^a	4–8
Negative (vehicle) control	4–8
Active Drug Dose 1	4–8
Active Drug Dose 2	4–8
Active Drug Dose 3	4–8

^a An appropriate duration of baseline data are collected immediately prior to each treatment condition. This data collection period may be utilized to positively establish signal quality and confirm animal/preparation suitability.

Dependent measures:

- Respiratory rate
- Tidal volume
- Minute volume
- Blood gas and co-oximetry measures[†]
- Clinical observations
- Body weight

[†] These and other parameters are particularly relevant in circumstances where a drug class effect or other specific concern may warrant evaluation.

EXPERIMENTAL DESIGN— RESPIRATORY SAFETY EVALUATION IN CONSCIOUS RODENTS OR GUINEA PIGS

Test system: 4–8 experimentally naïve or nonnaïve animals per treatment/group.*

Study design: The study is most commonly conducted according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route in animals that are either freely moving within a whole-body plethysmograph environment or restrained within a head-out or dual-chamber plethysmograph, articulated to a computerized data collection system capable of detecting and/or deriving a variety of essential parameters via raw pressure signals. Either independent group or within-subjects designs may be utilized, although independent group designs are more common. If conducted using a within-subjects design, dose administration typically proceeds according to a Latin square or ascending dose sequence, with an appropriate recovery period between treatments based on known PK/PD profiles. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound; due to the limited duration of recording, often up to a maximum of just several hours (head-out, dual-chamber restrained models), evaluation for metabolite effects may be more difficult in this context. Once animals are appropriately configured, a stable baseline facilitated by acclimation to ambient conditions is initially collected, followed by experimental treatments. Acclimation to the laboratory conditions when using a method of physical restraint often requires several days of procedural habituation prior to study initiation.

* Experimentally naïve or nonnaïve animals are considered acceptable, although naïve animals are more commonly utilized. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The rat and mouse are well-accepted species for which a large historical database is available; a somewhat lesser utilized model is the guinea pig.

Treatment/Condition	Number of Animals
Baseline ^a	4–8
Negative (vehicle) control	4–8
Active Drug Dose 1	4–8
Active Drug Dose 2	4–8
Active Drug Dose 3	4–8

^a An appropriate duration of baseline data are collected immediately prior to each treatment condition. This data collection period may be utilized to positively establish signal quality and confirm animal/preparation suitability.

Dependent measures:

- Respiratory rate
- Tidal volume
- Minute volume
- Blood gas and co-oximetry measures[†]
- Clinical observations
- Body weight

[†] These and other parameters are particularly relevant in circumstances where a drug class effect or other specific concern may warrant evaluation.

COMBINED CARDIORESPIRATORY SAFETY EVALUATIONS

Possibilities exist for temporally coordinated examination of standard cardiovascular and respiratory function variables presented in the previous sections, either in the context of a historically employed anesthetized preparation in a large animal test system, or more recently, by means of external or fully implanted telemetry devices. Technology enabling calibrated data collection within an anesthetized cardiorespiratory model has existed for decades, and generally allows for the greatest number and diversity of potential acquired end points. Recently developed telemetry units are equipped with multiple signal sensing components correspondingly capable of long-term monitoring of variables including respiratory and heart rates, tidal volume, blood pressure, electrocardiographic (or other) biopotentials, and ancillary physiological/behavioral parameters (body temperature, general activity) in large animal species.^{37,38} Although multiple and diverse telemetry-based systems, produced by several manufacturers exist, the selection of the technology utilized often depends upon many variables intrinsic to the individual laboratory, such as background experience and understanding of such applications, routine conditions of use, including study design characteristics, and other factors. As previously discussed for cardiovascular function testing, the main advantages of anesthetized cardiorespiratory preparations lie in the number of complex correlated variables that may be assessed to derive understandings of coordinated physiological functions. Although a more limited set of variables are accessible by

comparison, the main advantages of telemetry, in contrast to the anesthetized model, are the lack of potential confound related to use of (chemical) restraint, and the capacity to comonitor many variables with extremely high fidelity over extended periods of time, as is not generally feasible using alternative methods. General study designs intrinsic to either model are depicted here, along with selected corresponding data in the later sections.

Small animal test systems are generally equally amenable to evaluation by anesthetized cardiorespiratory models. Since a sample study design accordingly may be considered largely redundant by comparison to that conducted in large animals, a unified generic sample study outline is presented in this section which is applicable to either small or large animal species. In contrast to anesthetized models, creative arrangement of the environment in which telemetry-based and other systems may be applied in nonanesthetized small animals to measure cardiovascular and respiratory parameters is required to enable coordinated evaluations. There are numerous opportunities for synchronized assessment of a combination of relevant variables, as described in the literature,^{39,40} and as illustrated in the sample outline included in the table under “Experimental Design—Cardiorespiratory Safety Evaluation by Telemetry and Plethysmography in Conscious Rodents or Guinea Pigs.” CNS observational measures not requiring interaction with the test subject may also be employed at selected times in order to afford an additional behavioral/neurological characterization of potential test item-related effects. However, such CNS end points are likely more accessible and meaningful if applied in the context of whole-body plethysmography, as opposed to alternative plethysmography techniques (e.g., head-out) requiring restraint and which may tend to mitigate the capacity to fully observe the animal in a relatively more “naturalistic” laboratory environment.

EXPERIMENTAL DESIGN— CARDIORESPIRATORY SAFETY EVALUATION BY TELEMETRY IN CONSCIOUS NONRODENTS

Test system: 4–8 experimentally naïve or nonnaïve animals per treatment/group*

* Experimentally naïve or nonnaïve animals are considered acceptable. If nonnaïve animals are utilized, health status qualifications (clinical pathology, physical examinations, clinical observations, ECGs) are performed to permit assignment of individual animals to study. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The canine (beagle) and nonhuman primate (cynomolgus or rhesus monkey) are well-accepted species for which a growing historical database is available; a somewhat lesser utilized model is represented in swine (typically minipigs), although the telemetry technology is equally applicable across all of these species.

Study design: The study is most commonly conducted according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route in animals that are instrumented with calibrated telemetric devices and data are collected using associated software control systems capable of detecting and/or deriving a variety of cardiovascular and respiratory function parameters via raw pressure and biopotential signals. Either independent group or within-subjects designs may be utilized, although within-subjects designs are the most common approach. If conducted using a within-subjects design, dose administration typically proceeds according to a Latin square or ascending dose sequence, with an appropriate recovery period between treatments based on known PK/PD profiles. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound and metabolites, as applicable. Telemetry device implantation requires surgery and typically 10–14 days recovery prior to the initiation of data collection and dosing. Acclimation to the laboratory environment requires transfer of the animals to the study room at least 1 week prior to study initiation.

Treatment/Condition	Number of Animals
Baseline (24 h) ^a	4–8
Negative (vehicle) control	4–8
Active Drug Dose 1	4–8
Active Drug Dose 2	4–8
Active Drug Dose 3	4–8

^a An ~24 h period of baseline data is collected prior to dosing initiation to allow evaluation of telemetry signal quality, confirmation of animal suitability, and provision of an appropriate data record permitting establishment of individual animal QT/RR relationships for probabilistic QT interval correction.

Dependent measures:

- Systolic, diastolic, and mean arterial blood pressures
- Heart rate (derived from ECG or blood pressure signal)
- ECG: Quantitative intervals (RR, PR, QRS, QT, and QTc), qualitative assessment[†]

[†] Representative ECG tracings of appropriate duration (defined institutionally according to the goals of evaluation) are collected prior to exposure, at appropriate interval(s) postdose based on drug PK/PD characteristics, and near the end of the cardiorespiratory monitoring period for each treatment. Evaluation over these intervals permits an assessment of baseline, peak effect, and duration of action. ECG records are reviewed by a board-certified veterinary cardiologist, or otherwise qualified expert.

- Respiratory rate, tidal and minute volumes*
- Blood gas and co-oximetry measures[†]
- Ventricular pressure and derivatives (estimates of contractility, relaxation, etc.—optional)[‡]
- Body temperature, general activity (optional)[§]
- Clinical observations
- Body weights

* Respiratory inductance plethysmography (RIP) is available in either fully implantable configurations, or using a noninvasive approach. To monitor the full set of parameters listed earlier, a single implantable device may be utilized (e.g., DSI PCTR), or less ideally, combinations of implantable and noninvasive system components may be used.

[†] Blood gas analyses yield optional measures that may be considered in this design, as warranted based on individual study circumstances. Circumstances of data collection and experimental logistics should be considered carefully in relation to the known impact of such sampling procedures on physiological signals and behavior.

[‡] These parameters are accessible using standard available telemetry devices, and are particularly relevant in circumstances where a drug class effect may be of concern.

[§] Core body temperature requires appropriate placement of the temperature sensing component of the telemetry device; subcutaneous location of the device does not permit accurate recordings of core body temperature. Core temperature data are useful as a general indicator of potential CNS activity (as is general activity), as well as in circumstances where thermoregulation is affected (e.g., hypothermia) to the extent that correction of QTc values for body temperature is warranted.

EXPERIMENTAL DESIGN— CARDIORESPIRATORY SAFETY EVALUATION BY TELEMETRY AND PLETHYSMOGRAPHY IN CONSCIOUS RODENTS OR GUINEA PIGS

Test system: 4–8 experimentally naïve or nonnaïve animals per treatment/group*

Study design: The study may be conducted non-GLP, or according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Dosing is performed by the required route in animals that are instrumented with calibrated telemetric devices and placed within either a whole-body plethysmograph

* Experimentally naïve or nonnaïve animals are considered acceptable. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The mouse and rat represent appropriate models for blood pressure assessments in general. However, due to high resting heart rates and ion channels mediating repolarization, these species are not generally accepted models for definitive characterization of conductance and repolarization abnormalities (e.g., ICH S7B).

or alternative design (head-out, dual-chamber plethysmograph). Data are collected using associated software control systems capable of detecting and/or deriving a variety of cardiovascular and respiratory parameters via raw pressure and/or biopotential signals. Either independent group or within-subjects designs may be utilized. If conducted using a within-subjects design, dose administration typically proceeds according to a Latin square or ascending dose sequence, with an appropriate recovery period between treatments based on known PK/PD profiles. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound and metabolites, as applicable. Telemetry device implantation requires surgery and typically 7–14 days recovery prior to the initiation of data collection and dosing. Acclimation to the laboratory environment requires transfer of the animals to the study room at least 1 week prior to study initiation. Acclimation to the laboratory testing conditions when using a method requiring physical restraint often requires several days of procedural habituation prior to study initiation.

Treatment/Condition	Number of Animals
Baseline (24 h) ^a	4–8
Negative (vehicle) control	4–8
Active Drug Dose 1	4–8
Active Drug Dose 2	4–8
Active Drug Dose 3	4–8

^a A suitable period of baseline data may be collected prior to dosing initiation to allow evaluation of telemetry signal quality and confirmation of animal suitability. Telemetry collections of 24 h or more are easily accommodated by the technology; because of the issue of restraint, however, functional respiratory monitoring in a plethysmograph chamber is necessarily more limited.

Dependent measures:

- Systolic, diastolic, and mean arterial blood pressures
- Heart rate (derived from ECG or blood pressure signal)
- ECG: Quantitative intervals (RR, PR, QRS, QT, and QTc), qualitative assessment[†]
- Respiratory rate, tidal and minute volumes[‡]

[†] Collection of ECG data in guinea pigs for the purpose of evaluation for conductance and repolarization abnormalities is acceptable according to ICH S7B and contemporary scientific standards; mice and rats are not considered acceptable for modeling these effects.

[‡] Respiratory function parameters may be collected using a variety of commercially available plethysmography hardware and associated computerized control and recording systems.

- Blood gas and co-oximetry measures*
- Estimates of ventricular contractility and relaxation†
- Body temperature, general activity‡
- Clinical observations
- Body weights

* Blood gas analyses yield optional measures that may be considered in this design, as warranted based on individual study circumstances. This would most typically be performed in an appropriate number of satellite animals appropriately catheterized to allow arterial blood sampling at required intervals, and to mitigate the known impact of such sampling procedures on physiological signals and behavior.

† These parameters are accessible using standard available telemetry devices, and are particularly relevant in circumstances where a drug class effect may be of concern.

‡ Core body temperature requires appropriate placement of the temperature sensing component of the telemetry device; subcutaneous location of the device does not permit accurate recordings of core body temperature. Core temperature (as well as general activity) data are useful as a general indicator of potential CNS activity; temperature data sampling is also relevant in circumstances where thermoregulation is affected (e.g., hypothermia) to the extent that correction of QTc values for body temperature is warranted. Core temperature by appropriate placement of the telemetry device within the abdominal cavity is accessible in the guinea pig, but may be somewhat less so in the rat and mouse, depending on surgical approach and specific telemetry device employed.

EXPERIMENTAL DESIGN—ANESTHETIZED CARDIORESPIRATORY SAFETY EVALUATION

Test system: 3–6 experimentally naïve or nonnaïve animals per treatment/group*

Study design: The study may be conducted non-GLP, or according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability.

* Experimentally naïve or nonnaïve animals are considered acceptable, although nonnaïve animal utilization is somewhat less relevant to anesthetized preparations, as these often are terminal procedures. If nonnaïve animals are utilized (most typically for large animal species), particularly animals derived from telemetry stock colonies, health status qualifications (clinical pathology, physical examinations, clinical observations, ECGs) may be performed to permit assignment of individual animals to study. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., power analyses, historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication. The canine (beagle), rat, and guinea pig are well-accepted species for which a large historical database is available; lesser utilized models include nonhuman primate (cynomolgus or rhesus monkey), rabbits, swine (typically minipigs), marmosets, and ferrets. Due to an enhanced ability to control environmental variables and the range of exposures in especially anesthetized models (e.g., enhanced internal validity), fewer animals may be utilized in relation to designs employing unrestrained telemetric techniques in order to draw valid conclusions.

Dosing is performed by the required route in animals that are anesthetized and instrumented appropriately with calibrated devices for measuring desired parameters. Data are collected using associated software control systems capable of detecting and/or deriving a variety of cardiovascular and respiratory parameters via raw pressure and/or biopotential signals. Respiratory functions may be either assessed in spontaneously breathing subjects, or alternatively, if more specific measures (e.g., airway resistance, lung compliance, forced maneuvers) are required, in subjects on a mechanical ventilator. Either independent group or within-subjects designs may be utilized, although within-subjects designs are the most common approach, often with ascending/cumulative dosing regimens performed over several hours while animals are anesthetized. Data are recorded both immediately prior to, and following each exposure for a period of time appropriate to capture potential pharmacodynamic effects of the parent compound; due to the limited duration of recording, often up to a maximum of just several hours, evaluation for metabolite effects may be more difficult in this context. Once animals are appropriately instrumented, a stable baseline, facilitated by level surgical plane of anesthesia, is initially collected, followed by experimental treatments.

Treatment/Condition	Number of Animals
Baseline ^a	3–6
Negative (vehicle) control	3–6
Active Drug Dose 1	3–6
Active Drug Dose 2	3–6
Active Drug Dose 3	3–6

^a An appropriate duration of baseline data, often at least 0.5–1 h, is collected immediately prior to each treatment condition. This data collection period may be utilized to positively establish signal quality and confirm animal/preparation suitability.

Dependent measures:

- Systolic, diastolic, and mean arterial blood pressures
- Heart rate (derived from ECG or blood pressure signal)
- ECG: Quantitative intervals (RR, PR, QRS, QT, and QTc), qualitative assessment†

† Representative ECG tracings of appropriate duration (defined institutionally according to the goals of evaluation) are collected prior to exposure, at appropriate interval(s) postdose based on drug PK/PD characteristics, and near the end of the cardiovascular monitoring period for each treatment. Evaluation over these intervals permits an assessment of baseline, peak effect, and duration of action. ECG records are reviewed by a board-certified veterinary cardiologist, or otherwise qualified expert. Note that collection of ECG data in guinea pigs for the purpose of evaluation for conduction and repolarization abnormalities is acceptable according to ICH S7B and contemporary scientific standards; mice and rats are generally not considered acceptable by comparison for modeling these effects.

- Respiratory rate, tidal and minute volumes
- Blood gas and co-oximetry measures*
- Supplemental mechanical measures*
- Ventricular pressure and derivatives (estimates of contractility, relaxation, etc.)*
- Vascular compliance/resistance*
- Cardiac output*
- Estimates of myocardial work*
- Body temperature (optional)[†]
- Clinical observations (generally more applicable to nonanesthetized preparations)
- Body weights

* These and other parameters are particularly relevant in circumstances where a drug class effect may be of concern, and are generally more accessible in anesthetized models.

[†] Core body temperature data are useful as a general indicator of potential CNS activity, as well as in circumstances where thermoregulation is affected (e.g., hypothermia) to the extent that correction of QTc values for body temperature is warranted. During anesthetized procedures, core temperature should be monitored along with plane of surgical anesthesia in order to keep these regulated within a stable range; doing so will preempt the need for QTc correction for body temperature changes and may also help to mitigate heart rate variability. In cases where active drug treatments affect body temperature, or core temperature otherwise varies in an uncontrolled manner, having a sufficient number of temporally correlated QT/QTc and core body temperature values will allow QTc correction for such body temperature variations. Alternatively, correction factors have been proposed for various species and may be found in the scientific literature.

CENTRAL NERVOUS SYSTEM EVALUATIONS

As indicated in the prior sections detailing conduct of cardiovascular and respiratory functions, the purpose of the safety pharmacology core battery is to investigate the effects of the test substance on vital functions which are acutely critical for life. The central nervous system (CNS) represents a significant target for potential adverse drug action, particularly as it exerts executive and regulatory control over organic and coordinated system functions. As is true for approaches to other core battery cardio-respiratory assessments, there exists an almost limitless variety of assays that might be employed to evaluate potential off-target, neurobehavioral effects associated with drug administration. Accordingly, the approach to testing for potential CNS activity is similarly tiered in nature. Initial CNS safety evaluation procedures are primarily observational in nature, and measured outputs range from categorical discriminations (e.g., present/absent, or rank-ordered variables) to continuous, scaled

numerical values (e.g., body temperature, grip strength, open field rearing counts) which can be used to determine effects of concern, from the idiosyncratic, to those constellations of findings indicating more systematic change according to a broad functional domain of behavior and/or physiology.

In general, a functional observational battery (FOB), or similar multidimensional, scaled instrument (e.g., Irwin Screen) is an excellent method to approach the initial characterization of potential for either direct CNS activity or that mediated via secondary mechanisms (e.g., acute systemic toxicity).^{41–44} Such test batteries are useful because of the number and diversity of neurobehavioral status variables these assays are able to capture on a repeated basis, in addition to the flexibility of the instrument, which may be adapted to characterize species-specific manifestations of CNS drug action.^{42,43,45–47} Because detailed descriptions of these various screening methodologies are available in the literature, an exhaustive listing of parameters and procedures will not be characterized in this chapter. However, a representative sampling of relevant end points in a typical procedure⁴¹ is presented in Table 21.3 in relation to a depiction of essential (core battery) and supplemental measures indicated for various phases of CNS safety assessment (Table 21.4).

TABLE 21.3
Representative Functional Observational Battery
Assessments Routinely Conducted in Rodents

FOB (Continuous End Points) ^a	FOB (Categorical End Points) ^b	
Thermal response latency	Posture	Arousal
Mean forelimb grip strength	Ease of removal (home cage)	Vocalization
Mean hindlimb grip strength	Handling reactivity	Respiration
Mean hindlimb splay	Lacrimation	Stereotypy
Body temperature	Palpebral closure	Atypical behavior
Body weight	Piloerection	Approach response
Rearing	Exophthalmus	Touch response
Defecation	Salivation	Auditory stimulus Response
Urination	Clonic/tonic movements	Tactile (pinch) response
	Gait	Pupil response
	Mobility	Righting reflex

^a Parametric statistical analysis (e.g., ANOVA).

^b Nonparametric statistical analysis (e.g., Cochran Mantel Haenszel test).

TABLE 21.4
Examples of Core Battery (Tier I) and
Supplemental (Tier II) Central Nervous
System Function Measures Investigated in
Safety Pharmacology Studies

CNS Safety Pharmacology Measure	Core Battery (Tier I)	Supplemental (Tier II)
FOB/Irwin test battery	X	
Motor activity	X	
Ligand binding		X
Neurochemistry		X
Learning and memory assessments		X
Behavioral pharmacology/abuse and dependence liability assessments		X
Proconvulsant/seizure liability assessments		X
Special senses evaluations		X

EXPERIMENTAL DESIGN—CNS SAFETY EVALUATION IN NONRODENTS

Test system: 4–6 experimentally naïve or nonnaïve animals per treatment/group.*

Study design: The study is most commonly conducted according to GLP regulations, including dose formulation analysis for concentration, uniformity, and stability. Either independent group or within-subjects designs may be utilized, although these present with different issues regarding data collection and interpretation. With independent group designs, data derived from animals following active doses may be compared to the vehicle control, and/or to predose data collected within each treatment group.

* Experimentally naïve or nonnaïve animals are considered acceptable. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., most typically historical and/or positive control data references). Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication.

For within-subjects designs, utilization of a Latin square or similar randomization procedure should be standard practice to control for potential order effects related to learning (habituation) phenomena. Regardless of the specific approach, data are recorded prior to, and at selected intervals following each exposure for a number of times appropriate to capture potential pharmacodynamic effects of the parent compound and metabolites, but generally, at minimum, prior to dosing, at the time of expected peak effect (e.g., t_{\max}), and at an appropriate recovery interval to demonstrate potential for reversal (e.g., 24 h postdose).

Acclimation to the laboratory environment requires transfer of the animals to the study room at least 1 week prior to study initiation.

Treatment/Condition	Number of Animals
Negative (vehicle) control	4–6
Active Drug Dose 1	4–6
Active Drug Dose 2	4–6
Active Drug Dose 3	4–6

Dependent measures:

- Multiple measures sensitive to potential CNS or non-CNS-mediated activity, as indicated generally in Table 21.3, and more specifically as adapted to large animal species in alternative references^{46,47}
- Clinical observations
- Body weights

EXPERIMENTAL DESIGN—CNS SAFETY EVALUATION IN RODENTS

Test system: 6–10 experimentally naïve or nonnaïve animals per treatment/group.*

Study design: The study is most commonly conducted according to GLP regulations, including dose

* Experimentally naïve animals are more generally considered acceptable in comparison to nonnaïve animals. The number of animals utilized depends upon desired assay sensitivity, as established by institutional practice and appropriate modeling (e.g., most typically historical and/or positive control data references). In rodent test batteries, an $n = 10/\text{sex}/\text{group}$ has been established in prior regulatory guidelines (EPA, FDA) on behavioral neurotoxicity evaluation of chemicals and drugs. Positive control conditions may be included as necessary, but are not absolutely indicated on any individual study. Generally, male or female animals are equally acceptable, unless there is an expectation for a sexually dimorphic effect based on compound characteristics or therapeutic indication.

formulation analysis for concentration, uniformity, and stability. Either independent group or within-subjects designs may be utilized, although independent group designs are more common. With independent group designs, data derived from animals following active doses may be compared to the vehicle control, and/or to predose data collected within each treatment group. For within-subjects designs, utilization of a Latin square or similar randomization procedure should be standard practice to control for potential order effects related to learning (habituation) phenomena. Regardless of the specific approach, data are recorded prior to, and at selected intervals following each exposure for a number of times appropriate to capture potential pharmacodynamic effects of the parent compound and metabolites, but generally, at minimum, prior to dosing, at the time of expected peak effect (e.g., t_{\max}), and at an appropriate recovery interval to demonstrate potential for reversal (e.g., 24 h postdose).

Acclimation to the laboratory environment requires transfer of the animals to the study room at least 1 week prior to study initiation.

Treatment/Condition	Number of Animals
Negative (vehicle) control	6–10
Active Drug Dose 1	6–10
Active Drug Dose 2	6–10
Active Drug Dose 3	6–10

Dependent measures:

- Multiple measures sensitive to potential CNS or non-CNS-mediated activity, as indicated generally in Table 21.3.
- Clinical observations
- Body weights

INTEGRATED FUNCTIONAL SAFETY AND TOXICOLOGY STUDY DESIGNS

Safety pharmacology end points may be collected as indicated in the preceding sections, as stand-alone investigations, but increasingly are integrated to varying degrees within study designs traditionally utilized to characterize toxicity. For less invasive and/or sophisticated functional variables, this has been relatively readily achievable, but for others, integration has represented a challenge. Table 21.5

illustrates a variety of core battery safety pharmacology measures and the current proposed status as an integrated variable. With time and technological developments have come increased opportunities for reconciling the design characteristics appropriate for the somewhat disparate goals of safety pharmacology and toxicology testing. While the integration of safety pharmacology and toxicology represents a potential opportunity for resource savings (e.g., realization of 3 R's principles), and has been encouraged by regulatory experts,^{4,5,16–18} there are a variety of factors which must be carefully considered in arriving at an acceptable final study design that will add value to the process. There may exist circumstances under which it is not technically feasible or scientifically justifiable to integrate certain functional (safety pharmacology) variables into a larger design. In this regard, two questions are of primary importance in as much as these may be used to guide appropriate program development: (1) for what essential purpose is the functional measure being added? and (2) particularly in situations where regulatory need (ICH S7A, ICH S7B) is cited, is the choice of end points for assessment, and approach to data collection procedurally consistent with current industry scientific and regulatory best practices? In general, a useful guiding principle is to avoid situations where appropriate assay sensitivity and specificity may be unacceptably compromised due to such factors as the introduction of uncontrolled environmental influences or adoption of less than adequate technology. In illustration of this point (Figure 21.1), it has repeatedly been demonstrated that the operating characteristics, including susceptibility to signal disruption or limited sampling rate, of different electrocardiographic data collection procedures have a fundamental influence on sensitivity to characterize quantitative or qualitative changes of interest. This is a convenient example of a general concern which applies broadly to all efforts to identify potential pharmacologically mediated effects on sensitive behavioral and physiological end points which are the focus of safety pharmacologists. Although it is not always possible to perfectly emulate the “gold standard” study design characteristics used in stand-alone safety pharmacology experiments, care should be exercised to take all reasonable steps in creating an environment conducive to maximizing similarity to that ideal as reflected in current industry best practices.^{20,27} In so doing, we may realize significant added value in terms of enhanced ability to characterize potential liabilities, while avoiding the pitfall of generating questionable quality data that may promote misleading interpretations.⁴⁸

Opportunities for study design integration are potentially available for almost any functional safety variable that one has concern to investigate. Accordingly, specific examples of such hybrid safety pharmacology/toxicology study designs will not be presented herein. However, it is

relatively easy to envision that most circumstances involve reconciliation of the contextual application of a selected measurement technique (equipment, procedures, testing environment, etc.) with the primary goals of safety pharmacology studies to characterize often acute, off-target and potentially adverse pharmacology in a manner consistent with principles of dose–response analysis, including elucidation of mechanism(s) of action, and potential reversibility. Often procedures facilitative of functional evaluation in one species are amenable to adaptation in order to allow similar application in other species that may represent a more appropriate test system for the particular molecule under consideration. For example, the utilization of FOB testing routines depicted in the table under “Experimental Design—CNS Safety Evaluation in Nonrodents” for large animals, including the nonhuman primate, may be successfully integrated as periodic behavioral assessments for potential adverse CNS-mediated activity within a necessary repeated dosing toxicology study. Depending on the particular circumstances, involved behavioral monitoring conducted in order to more specifically characterize potential neurobehavioral adverse effects (habituation learning, memory, dependence liability, etc.) may also be relatively easily scheduled in the context of repeated dose studies. Similarly, opportunities for evaluation of respiratory and cardiovascular functions, while requiring specialized equipment, are often relatively readily integrated into repeated dose toxicology designs, simply requiring a more controlled environment often for a cohort of designated animals to be intensively monitored over an appropriate interval in relation to dose administration(s). There currently exist several alternatives for cardiorespiratory monitoring, as indicated in previously presented study design examples, which all afford significant gains in terms of sensitivity to potential effects of concern in relation to improvements in signal quality and continuity and density of signal/data sampling in relation to historical methodologies. As these examples illustrate, various and diverse approaches to conducting such integrated evaluations are under consideration; with time, some enhanced degree of uniformity regarding generalized “best practices” will likely emerge. However, even with the eventual establishment of standard approaches to incorporating new and superior technologies to assess functional biomarkers in the toxicology study context, individual circumstances of each development program will require careful thought toward the generation of unique solutions in achieving programmatic success.

TABLE 21.5
A Contemporary Assessment of the Status of Various Functional (Safety Pharmacology) Parameters Relative to Perceived Opportunities for Integration within Toxicology Study Designs

Parameter	Safety Pharmacology	Rodent Toxicology	Nonrodent Toxicology
CNS safety			
Functional observational battery	+++	++/+++	+/++
Cardiovascular safety			
Electrocardiogram	+++	+/++	+++
Blood pressure	+++	+/++	+/++
Ventricular pressure	+++	+/++	+/++
Respiratory safety			
Inspiratory/expiratory time	+++	+/++	+/++
Peak inspiratory/expiratory flow	+++	+/++	+/++
Respiratory rate	+++	+/++	+/++
Tidal volume	+++	+/++	+/++
Pulmonary resistance and compliance	+/++	–/+	–/+
Hyperreactivity	+/++	–/+	–/+

Notes: A contemporary assessment of the status of various functional (safety pharmacology) parameters relative to perceived opportunities for integration within toxicology study designs. This table represents an adaptation from Gad et al. (2004) based on shifts in regulatory thought, evolving scientific best practices, and technological innovation. Some opportunities for integration, such as evaluation of respiratory volume and rate in rodents, merely have required the formalization of thoughts regarding how best to embed the utilization of various available plethysmography devices into the context of a rodent toxicology study design, taking care to address issues of laboratory and device acclimation, appropriateness of selected dose levels, and timing of evaluations in relation to other ongoing (toxicology) data collections. Other opportunities for integration, such as evaluation of quantitative and qualitative cardiovascular safety measures in large animal (nonrodent) toxicology study designs, and in a manner considered ideal by contemporary science and regulatory guidance, have required the development of technological innovations in noninvasive and implantable telemetry, with corresponding time for experimentation with such technology in different experimental circumstances to eventually arrive at procedures which may approximate industry “best practices.” Some functional measurements, including such examples as sophisticated behavioral testing routines (operant behavior), mechanistic cardiovascular investigations into myocardial work or related parameters, and the evaluation of pulmonary resistance/compliance measures, are relatively more challenging to approach in the context of the integrated safety/toxicology study design, and indeed, for the present, may remain best served by independent, focused studies. +++, Already integrated; ++, integration after adaptation easy; +, integration after adaptation possible; –, integration not possible; slashed indications indicates status falls between the symbols shown.

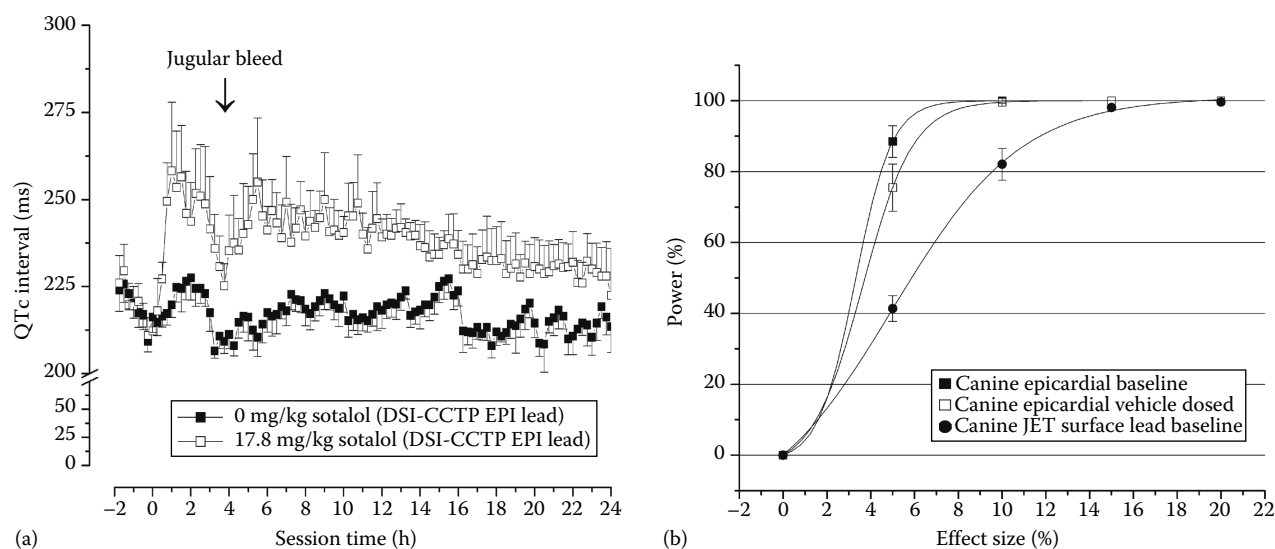


FIGURE 21.1 Two examples of environmentally and methodologically induced variability, with potential to influence assay sensitivity. The Figure 21.1(a) illustrates the cardiovascular response to a dose of the Class III antiarrhythmic drug, sotalol hydrochloride. Electrocardiographic (ECG) biopotentials were collected in animals instrumented with fully implanted telemetry transmitters (DSI D70-CCTP) configured with modified base-apex epicardial lead array. Note the masking of QTc increase during the period of expected peak drug effect that is induced by handling of the animal for collection of a jugular blood sample. The data illustrate the dramatic influence of salient environmental stimuli often associated with the toxicology study milieu on a key functional safety parameter, as well as the utility of continuous data recording afforded by telemetry toward effectively characterizing such changes in contrast to historically employed, limited sampling techniques. The Figure 21.1(b) illustrates the results of a statistical power analysis conducted in three alternative datasets (~24 h in duration each) derived from differentially collected ECG signals in beagle dogs: (1) standard intrathoracic base-apex epicardial lead configuration in an undisturbed laboratory environment, (2) standard intrathoracic base-apex epicardial lead configuration in a laboratory environment where animals were handled and dosed with an inert vehicle, and (3) standard Jacketed External Telemetry (JET) skin surface lead configuration in an undisturbed laboratory environment. In all three conditions, power curves were estimated based on 1000 simulations and for an $n = 8$, with the resulting functions between effect size and power estimate. From the generated curves, two things are clear. First, comparing conditions 1 and 2, the influence of the effect of handling and oral gavage dosing of an inert vehicle is discernable from the undisturbed laboratory condition, resulting in decreases in the effect size that may be detected at a given level of power. Second, the influence of recording methodology (condition 1 versus condition 3) has an even greater impact on experimental power. While the power to detect a given magnitude of effect is decreased using JET-based technology compared to the “gold standard” epicardial lead derived from fully implantable telemetry technology, it should be noted that sensitivity associated with recently developed JET-based technology is still significantly better than historical methods of ECG collection employing vastly inferior sampling rates and requiring mechanical restraint routinely associated with increased artifact and physiological variability.

STUDY IMPLEMENTATION

Implementation of a successful safety pharmacology program relies upon the proper consideration of a variety of factors with considerably variable relevance to, and accordingly potential impact on, the series of unique study design options ultimately adopted based on individual circumstances surrounding disparate categories of molecules. For early (so-called “front-loaded”) safety pharmacology studies, which often involve screening for potentially insurmountable functional safety issues to prioritize compounds and/or engineer classes of molecules away from key liabilities,⁵⁰ the availability of early safety and exposure data are relatively less important than for the GLP designs employed to definitively characterize core

battery safety parameters. Intrinsic properties of the molecule, including broad versus narrow activity, substrate targeting, exposure and potency by species, and a host of other considerations, are among the primary influences guiding safety pharmacology study design and conduct. In some cases, as discussed in the preceding section, situational variables are conducive to the conduct of integrated toxicology and safety pharmacology study designs; in other cases, stand-alone studies represent the best option for managing divergent goals of the toxicology and safety pharmacology programs. In either case, there are choices regarding practical and logistic considerations that must be managed appropriately. In the sections immediately to follow are brief considerations of some of the most important determinants of safety pharmacology study design

and conduct, which, if given the appropriate consideration, will allow for the successful planning and execution of requisite functional investigations.

COMPOUND CHARACTERISTICS

Just as is true for toxicological profiling, the nature and scope of a safety pharmacology program depends significantly on the type of molecule being evaluated. It is quite clear that the safety pharmacology harmonized (ICH S7A, S7B) guidelines were written with considerable influence from the small molecule development perspective, and accordingly must be utilized as a generalized reference, particularly in circumstances where novel, nonsmall molecule therapeutics (monoclonal antibodies, vaccines, etc.) are under investigation. As a general rule, small molecules that are intended for chronic administration for indications other than life-threatening conditions often require the most intensive safety pharmacology programs in terms of number and variety of requisite studies. As the molecule moves in the opposite direction on these two dimensions (decreased chronicity of dosing, enhanced urgency of medical need), it is increasingly typical for the scope of the corresponding safety pharmacology package to be reduced and/or for key safety pharmacology biomarkers to be integrated within the context of (often repeated dose) toxicology study designs. Whether integration is a realistic opportunity, of course, depends upon compatibilities on such variables as dose and species selection, environmental factors, technical considerations, and basic logistical concerns as these relate to proper study execution.

In general, for most small molecules that are intended to be administered on a repeated basis, a minimum core battery safety pharmacology program will be comprised of an assessment of cardiac potassium channel interaction (e.g., hERG), as well as CNS and cardiorespiratory testing with previously specified “Tier I” evaluations (Tables 21.1, 21.2, and 21.4) incorporated into constituent study designs.⁵¹ For many small molecules, but particularly for those with a temporally limited exposure and/or pharmacodynamics, stand-alone *in vivo* studies to assess potential cardiovascular, respiratory, and CNS effects may be the best option, due to the relatively limited window of opportunity present to adequately characterize potential adverse pharmacology. Logistical considerations, particularly as competing evaluative procedures may have potential to adversely impact safety pharmacology assay sensitivity, are key considerations for such molecules. However, as therapeutic (and suprathreshold) exposure windows expand, as variably may be the case for biotechnology-derived therapies such as monoclonal antibodies, proteins, polypeptides, vaccines, oligonucleotides, etc., the concerns associated with potential adverse impact of study logistics also tends to be mitigated, and recognition of

opportunities for scientifically justifiable incorporation of core battery safety pharmacology evaluations into larger safety designs becomes much more feasible. There are other considerations relevant to biotechnology-derived pharmaceuticals which also may tend to complicate pharmacological (and general safety) characterization that are discussed in detail elsewhere.⁵² The issue of strict feasibility ultimately becomes a secondary consideration in whether and how to include functional safety assessments within the safety program—the main consideration with such targeted therapies is whether there is a rational basis to believe that incorporation of any such assessments, often being directly relevant to the molecule’s primary therapeutic activity, adds value to the quality of data developed toward better characterizing risk to allow more effective clinical management.

PREREQUISITE DATA

Safety pharmacology studies, and particularly those core *in vitro* and *in vivo* designs evaluative of cardiorespiratory and CNS functions, are often conducted early in the process of preclinical drug safety testing. The occurrence of these at an early point in the process is thought to serve at least two purposes. First, it may afford early identification of potentially unacceptable risk profiles on critical organ/system functions so that resources may be effectively managed toward more clinically acceptable molecules among multiple candidates. Second, this approach may ultimately reduce molecule attrition by affording the opportunity to characterize unacceptable safety profiles and associated mechanisms of action, allowing one to engineer discovery programs in more palatable directions with regards to managing risk.⁵⁰

Although benefits beyond these may be realized by “front-loading” safety pharmacology in such manner, it is also true that there are several critical pieces of information that should ideally have been developed *a priori* in order to maximize the efficiency and potential value of particularly the definitive GLP safety pharmacology program ultimately undertaken. These include information that may be gleaned from *in vitro* activity screening (ion channel, receptor interactions), as well as early pharmacokinetic, maximum tolerated dose, and early repeated dose toxicology studies, which may be utilized to inform on issues related to dose selection, species and/or assay selection, and potential categories of adverse effect in relation to anticipated exposures. By far, the conduct of appropriately timed, routine clinical observations in such studies, where applicable, is one of the most effective means of developing data critically useful to defining some of the most essential characteristics of the safety pharmacology program for a given molecule. Having indicated these primary sources of prerequisite data, it should be acknowledged that often

information from longer-term toxicity studies may importantly influence individual decisions and overall strategies for comprehensive safety pharmacology testing, particularly as these relate to dosage selection and the need for characterization of functional changes associated with chronic dosing protocols.

TEST SYSTEM AND DOSE SELECTION

For any given model, including those used to predict possible safety pharmacology concerns, several features are justifiably critical prerequisites to broad scientific acceptance. These include multiple factors which influence two key areas of concern—reliability and validity. In the present context, reliability may be defined as the extent to which various safety pharmacology preclinical testing methods are able to yield consistent or stable results, or are free of excessive (random) error variance. Validity may be defined essentially as the extent to which results of a safety pharmacology experiment are (1) due to specific manipulations of the independent variable, rather than some other systematic influence related to a weakness in experimental design (e.g., internal validity) and (2) able to be generalized, or are applicable to subjects and settings beyond the individual experiment (e.g., external validity). Test system selection may influence both the reliability and validity of a given model, and it is up to the experimenter to determine the relative risks to each, as these are influenced by the specific approaches to implementation of safety pharmacology assays in various species within their laboratory setting. Interacting with test system influences on model reliability and validity are technical, environmental, and procedural variables, which are discussed in the following section.

In terms of the focus of this section, an obvious practical impact of the test system may lie in whether the species utilized in the preclinical safety pharmacology study possesses key molecular targets of the candidate pharmacotherapy under investigation. For example, and as noted in the tables presenting rodent cardiovascular study design options, both internal and external validity may be adversely impacted by the relative insensitivity of a rat model to repolarization reserve shifts associated with drug treatment due to a fundamental species-related difference in the ion channels responsible for such repolarization. In similar manner, both reliability and (particularly external) validity may be adversely impacted due to selection of an inappropriately constrained range of drug doses in a given safety pharmacology study. Examples such as these are numerous, and highlight the importance of species and dose selection in terms of the influence on reliability and validity of safety pharmacology studies. Three of the most compelling considerations in species selection relate to (1) presence of the molecular target(s) in the test species, (2) other intrinsic

properties of the species determinative of experimental reliability and validity, and (3) prior experiences with the potential test system, including those occurring at earlier stages of the development program, which may corroborate or contraindicate utilization of that species.

By both historical and regulatory definitions, as well as contemporary understandings of professional practitioners, safety pharmacology studies are intended to evaluate potential dose- and time-dependent adverse pharmacology in the predicted therapeutic range and above. As standard practice, current protocols contain specific justifications of the rationale for dose selection, often based on the integrated pattern of results from prior investigations of exposure, acute behavioral and clinical observations, and dose-limiting adverse effect(s) and/or toxicity. Often, the challenge of setting the dose range for a safety pharmacology investigation is related to appropriate identification of the maximum dose to be evaluated. This may be due either to a lack of activity up to very high dose levels producing correspondingly extremely exaggerated systemic exposures compared to those associated with the known or predicted therapeutic dose level, or because of excessive activity at relatively low dose levels approximating the therapeutic range. In the first case, there is often no reasonable rationale for limiting doses to an arbitrary low multiple of the anticipated therapeutic dose, although nor may there be a practical opportunity to elicit dose–response pharmacology. In such cases, limit dose testing may represent a viable experimental design strategy. In the second case, the challenge is selecting a high dose which approximates a maximum tolerated dose, but which does not possess liability to induce systemic toxicity which secondarily recruits changes in measured functional parameters which are not mediated through receptor-relevant pharmacological mechanisms. In such circumstances, careful consideration must be given to identifying the most appropriate model for functional safety testing. As was indicated previously in outlines detailing anesthetized cardiorespiratory testing strategies, a potential benefit of utilizing this methodology lies in the ability to extend the dose range in a manner not possible in alternative (conscious, telemeterized) models. Although current regulatory guidance suggests a bias toward utilization of telemetry-based models and away from the use of physical/chemical restraint, this concern must be weighed against the objective realities of the circumstances; in many cases, the most relevant and useful information pertaining to critical safety parameters may, in fact, be gained from such historically validated anesthetized models.

ENVIRONMENTAL CONTROLS AND ASSAY SENSITIVITY

The prior discussion of species and dose selection are inevitably interrelated to considerations of the influence

of two additional factors heavily influencing experimental reliability and validity. As illustrated previously in Figure 21.1, specific choices made regarding the technical conduct of the study may either enhance or diminish overall model stability and generalizability. An experimental design and associated statistical routines with characteristics promoting the appropriate balance of reliability and (internal) validity may be described as possessing appropriate power to detect changes in the dependent variable properly attributable to the independent variable. In the context of a safety pharmacology study, this may represent, for example, the ability to correctly conclude that there is a causal relationship between the dose of drug administered and the elicitation of electroencephalographic biomarkers for proconvulsant activity. In an alternative example specifically depicted in Figure 21.1(a), this would represent the capacity to correctly reject the null hypothesis that sotalol administration (17.8 mg/kg, p.o.) was not associated with an increase in a key biomarker of repolarization reserve,

the QTc interval duration. One of the most significant threats to the ability to correctly reject the null hypothesis, or differently stated, the inability to establish an association between manipulation of the independent variable and measured aspects of the dependent variable, is low power. There are a variety of ways to enhance assay sensitivity to increase statistical power, facilitating appropriate detection of the influence of the independent variable on the dependent variable, including (1) increasing the number of experimental subjects, (2) increasing the duration of data sampling, (3) increasing the density of data sampling, (4) ensuring sound practices in regards to signal derivation and/or processing, and (5) instituting environmental controls on extraneous variables related to factors such as species selection, housing, acclimation, technology utilization, and other laboratory-based variables. See Figure 21.2 for a comparison of the relative influence of manipulation of two of these factors—number of subjects, and technique for ECG recording—on experimental power.

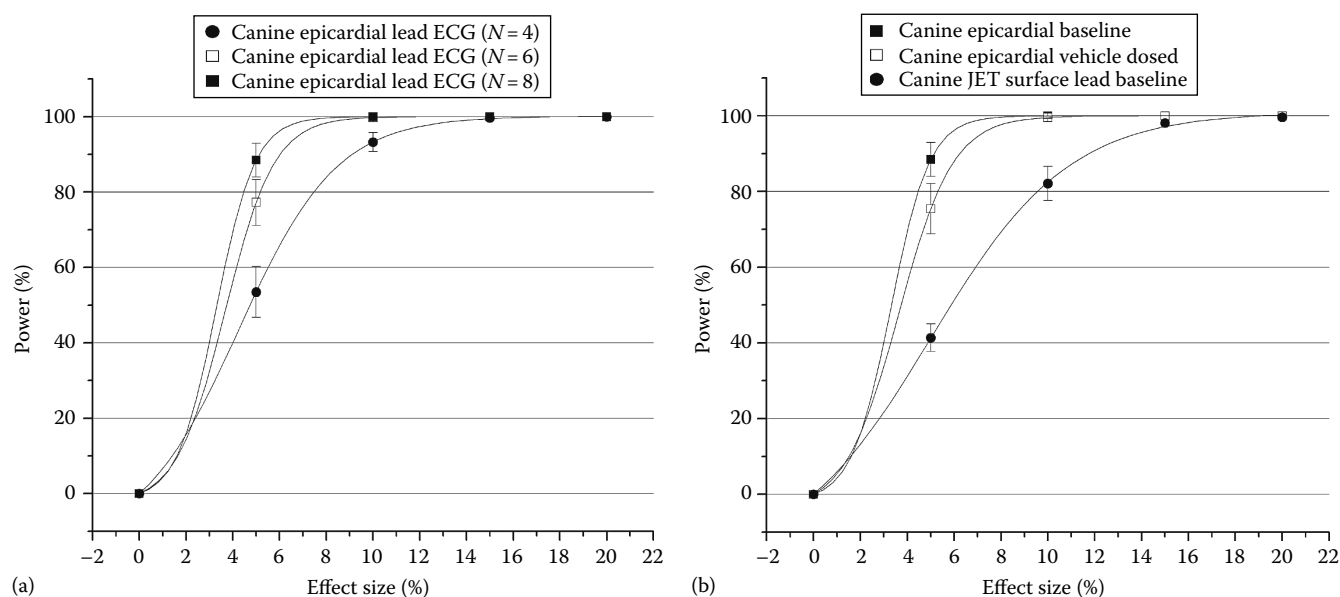


FIGURE 21.2 Comparison of the relative influence of two study design variables on experimental power. (a) The influence of statistical power modeling with assumptions of differential N within any individual experiment. Power curves were derived for the indicated number of subjects based on 1000 simulations conducted using baseline telemetry data. (b) (derived in similar manner and presented previously, see Figure 21.1 for details) Depicts the impact of the specific recording technique/condition utilized on experimental power. Both variables are clearly influential to a significant degree, and with other experimental model characteristics, must be considered carefully to achieve an optimum balance of internal and external validity.

HISTORICAL CONTROL DATA FOR CORE SAFETY PHARMACOLOGY ASSAYS

The data depicted in the following tables are generally consistent with application of the previously illustrated experimental designs (cardiovascular telemetry, cardiorespiratory telemetry, whole body and restrained plethysmography, CNS evaluations) in nonrodents and rodents, as applicable. Data are presented in each table in a standardized format, with descriptive statistics characterizing the essential parameters reported. Note that means, standard deviations, and ranges most typically reflect continuous data collection

(e.g., cardiovascular telemetry and respiratory plethysmography), often over extended periods of time with high temporal fidelity and density of sampling, and accordingly, are characterized by a range of circadian and ultradian variation that may not be typical in models not incorporating a similar breadth and depth of physiological sampling. The presentation of CNS test battery (categorical) data relies upon a convention relating a given measurement scale for an individual test item to a graphic illustration of the distribution of scores accumulated across the range of values on that scale (Tables 21.6 through 21.23).

TABLE 21.6
Cardiovascular Parameters Collected by Implanted Telemetry in the Beagle Dog

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Systolic arterial pressure (mm Hg)	174	140	17	86	210
Diastolic arterial pressure (mm Hg)	174	74	12	30	140
Mean arterial pressure (mm Hg)	174	98	14	51	160
Heart rate (beats/min)	174	84	19	38	180
PR interval (ms)	174	100	15	67	160
QRS interval (ms)	174	43	4.6	18	64
QT interval (ms)	174	230	18	150	310
QT interval—corrected (ms)	174	230	14	170	300
Body temperature (°C)	174	37	1.1	33	40
Female					
Systolic arterial pressure (mm Hg)	40	130	15	89	190
Diastolic arterial pressure (mm Hg)	40	73	11	38	110
Mean arterial pressure (mm Hg)	40	96	12	59	140
Heart rate (beats/min)	40	87	19	47	170
PR interval (ms)	40	110	13	71	150
QRS interval (ms)	40	45	4.6	33	68
QT interval (ms)	40	220	14	170	280
QT interval—corrected (ms)	40	220	8.6	190	260
Body temperature (°C)	40	36	1.3	33	39

Note: Statistics were generated from ~24 h recording intervals in male and female beagle dogs between 6 months and 4 years of age.

TABLE 21.7
Cardiovascular Parameters Collected by Implanted Telemetry
in the Nonhuman Primate (Cynomolgus Macaque)

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Systolic arterial pressure (mm Hg)	91	94	14	54	150
Diastolic arterial pressure (mm Hg)	91	63	11	26	100
Mean arterial pressure (mm Hg)	91	79	12	42	120
Heart rate (beats/min)	91	120	33	56	250
PR interval (ms)	91	86	14	48	150
QRS interval (ms)	91	38	4.6	20	50
QT interval (ms)	91	250	45	120	380
QT interval—corrected (ms)	91	250	32	150	340
Body temperature (°C)	91	38	0.79	35	40
Female					
Systolic arterial pressure (mm Hg)	41	97	12	70	160
Diastolic arterial pressure (mm Hg)	41	65	11	35	110
Mean arterial pressure (mm Hg)	41	81	11	53	130
Heart rate (beats/min)	41	140	34	72	280
PR interval (ms)	41	82	8.3	59	110
QRS interval (ms)	41	37	3.9	25	51
QT interval (ms)	41	240	44	130	350
QT interval—corrected (ms)	41	240	29	150	330
Body temperature (°C)	41	38	0.89	35	40

Note: Statistics were generated from ~24 h recording intervals in male and female cynomolgus monkeys between 2 and 5 years of age.

TABLE 21.8
Cardiovascular Parameters Collected by Implanted Telemetry in the Gottingen
Minipig

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Systolic arterial pressure (mm Hg)	18	140	14	110	180
Diastolic arterial pressure (mm Hg)	18	95	14	67	140
Mean arterial pressure (mm Hg)	18	120	13	88	160
Heart rate (beats/min)	18	93	15	56	180
PR interval (ms)	18	110	12	75	150
QRS interval (ms)	18	40	7.1	28	80
QT interval (ms)	18	300	27	220	380
QT interval—corrected (ms)	18	300	20	250	360
Body temperature (°C)	18	38	0.80	35	40
Female					
Systolic arterial pressure (mm Hg)	24	140	17	94	190
Diastolic arterial pressure (mm Hg)	24	96	14	45	140
Mean arterial pressure (mm Hg)	24	120	15	77	170
Heart rate (beats/min)	24	96	16	51	170
PR interval (ms)	24	100	9.3	78	140
QRS interval (ms)	24	38	5.6	25	55
QT interval (ms)	24	290	26	210	360
QT interval—corrected (ms)	24	290	24	230	360
Body temperature (°C)	24	38	0.80	35	40

Note: Statistics were generated from ~24 h recording intervals in male and female minipigs between 6 months and 2 years of age.

TABLE 21.9
Cardiovascular Parameters Collected by Implanted Telemetry in the Rat

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Systolic arterial pressure (mm Hg)	15	120	9.5	100	160
Diastolic arterial pressure (mm Hg)	15	85	7.7	70	120
Mean arterial pressure (mm Hg)	15	100	8.2	84	140
Heart rate (beats/min)	16	400	44	310	490
PR interval (ms)	6	43	3.4	37	55
QRS interval (ms)	6	24	1.8	21	29
QT interval (ms)	6	76	12	54	100
Body temperature (°C)	6	38	0.56	36	39

Note: Statistics were generated from ~24 h recording intervals in male and female rats 8–12 weeks of age.

TABLE 21.10
Cardiovascular Parameters Collected by Implanted Telemetry in the Mouse

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Systolic arterial pressure (mm Hg)	5	130	22	93	210
Diastolic arterial pressure (mm Hg)	5	110	22	66	190
Mean arterial pressure (mm Hg)	5	120	24	73	200
Heart rate (beats/min)	6	610	91	320	870
PR interval (ms)	6	31	3.9	23	48
QRS interval (ms)	6	15	2.3	12	20
QT interval (ms)	6	47	7	27	69
Body temperature (°C)	6	33	1.3	29	36

Note: Statistics were generated from ~24 h recording intervals in male mice 8–14 weeks of age.

TABLE 21.11
**Respiratory Function Parameters Collected by Head Plethysmograph
 in the Beagle Dog**

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Respiratory rate (breaths/min)	42	73	66	11	290
Tidal volume (mL)	42	160	110	19	490
Minute volume (mL/min)	42	7900	5500	1700	25,000
Female					
Respiratory rate (breaths/min)	28	58	56	11	270
Tidal volume (mL)	28	110	41	27	250
Minute volume (mL/min)	28	4000	2000	1000	8,500

Note: Statistics were generated from recording intervals of at least 1 h duration in male and female beagle dogs between 6 months and 4 years of age.

TABLE 21.12
Respiratory Function Parameters Collected by Head Plethysmograph
in the Nonhuman Primate (Cynomolgus Macaque)

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Respiratory rate (breaths/min)	58	42	9.4	24	71
Tidal volume (mL)	58	28	8.8	8.6	65
Minute volume (mL/min)	58	1200	510	220	2800
Female					
Respiratory rate (breaths/min)	40	38	9	23	67
Tidal volume (mL)	40	24	6.2	11	39
Minute volume (mL/min)	40	920	380	360	2400

Note: Statistics were generated from recording intervals of at least 1 h duration in male and female monkeys between 2 and 5 years of age.

TABLE 21.13
Respiratory Function Parameters Collected by Impedance
Plethysmography (Telemetry) in the Beagle Dog

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Respiratory rate (breaths/min)	5	27	15	7.9	82
Tidal volume (mL)	5	127	52	36	440
Minute volume (mL/min)	5	3800	3700	560	25,000

Note: Statistics were generated from ~24 h recording intervals in unrestrained, freely moving male and female beagle dogs between ~7 and 10 months of age.

TABLE 21.14
Respiratory Function Parameters Collected by Impedance
Plethysmography in the Nonhuman Primate (Cynomolgus Macaque)

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Respiratory rate (breaths/min)	5	24	6.1	13	47
Tidal volume (mL)	5	15	6.3	4	39
Minute volume (mL/min)	5	370	180	87	1700

Note: Statistics were generated from ~24 h recording intervals in unrestrained, freely moving female cynomolgus monkeys between ~3.5 and 5.5 years of age.

TABLE 21.15
Respiratory Function Parameters Collected by Impedance
Plethysmography in the Gottingen Minipig

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Respiratory rate (breaths/min)	5	23	11	7	63
Tidal volume (mL)	5	85	24	33	190
Minute volume (mL/min)	5	1800	850	500	6800

Note: Statistics were generated from ~24 h recording intervals in unrestrained, freely moving male Gottingen minipigs between ~7 and 10 months of age.

TABLE 21.16
Respiratory Function Parameters Collected by Whole-Body
Plethysmograph in the Sprague-Dawley Rat

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Respiratory rate (breaths/min)	621	140	45	54	430
Tidal volume (mL)	621	1.1	0.31	0.34	3.1
Minute volume (mL/min)	621	140	36	47	530
Female					
Respiratory rate (breaths/min)	80	130	51	64	360
Tidal volume (mL)	80	1.1	0.24	0.41	2.2
Minute volume (mL/min)	80	120	30	32	310

Note: Statistics were generated from recording intervals of at least 1 h duration in male and female rats between 7 and 10 weeks of age.

TABLE 21.17
Respiratory Function Parameters Collected by Head-Out
Plethysmograph in the Sprague-Dawley Rat

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Male					
Respiratory rate (breaths/min)	22	140	38	77	280
Tidal volume (mL)	22	1.2	0.43	0.44	3.4
Minute volume (mL/min)	22	160	60	51	500
Female					
Respiratory rate (breaths/min)	24	130	42	67	250
Tidal volume (mL)	24	1.1	0.17	0.56	1.4
Minute volume (mL/min)	24	137	37	61	270

Note: Statistics were generated from recording intervals of at least 1 h duration in male and female rats between 7 and 10 weeks of age.

TABLE 21.18
Respiratory Function Parameters Collected by Whole-Body
Plethysmograph in the CD-1 Mouse

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Respiratory rate (breaths/min)	56	310	110	140	570
Tidal volume (mL)	56	0.21	0.039	0.11	0.37
Minute volume (mL/min)	56	52	22	21	120

Note: Statistics were generated from recording intervals of at least 1 h duration in male and female mice between 7 and 10 weeks of age.

TABLE 21.19
Respiratory Function Parameters Collected by Whole-Body
Plethysmograph in the Guinea Pig

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Respiratory rate (breaths/min)	8	96	21	63	190
Tidal volume (mL)	8	3.9	0.45	2.3	5.3
Minute volume (mL/min)	8	350	54	220	550

Note: Statistics were generated from recording intervals of at least 1 h duration in male Crl:HA (Albino Hartley) Guinea pigs between 1 and 3 months of age.

TABLE 21.20
Central Nervous System Function Parameters in the Beagle Dog



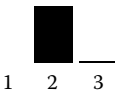



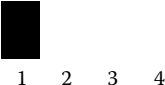
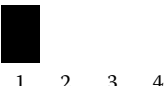






















Observation	Number of Observations	Assessment Range	Distribution
Categorical Variables			
<i>Activity/Arousal</i>			
Ease of removal	91	1 (very easy), 2, 3, 4, 5 (very difficult)	
General arousal	91	1 (very depressed), 2, 3, 4 (normal), 5, 6 (very elevated)	
Handling reactivity	91	1 (very low), 2 (low), 3, 4, 5 (high)	
Posture	91	1 (normal), 2, 3, 4 (bizarre/abnormal)	
<i>Autonomic</i>			
Exophthalmus	91	1 (not present), 2 (present)	
Lacrimation	91	1 (none), 2, 3 (severe)	
Palpebral closure (handling)	91	1 (eyelids wide open), 2, 3, 4 (eyelids completely shut)	
Palpebral closure (home cage)	91	1 (eyelids wide open), 2, 3, 4 (eyelids completely shut)	
Philoerection	91	1 (not present), 2 (present)	
Pupil size	91	1 (no noticeable constriction or dilation), 2, 3 (marked constriction or dilation)	
Pupil response	91	1 (normal pupil constriction), 2, 3 (lack of pupil constriction)	
Salivation	91	1 (none), 2, 3 (severe)	
<i>Neuromuscular</i>			
Bizarre behavior	91	1 (not present), 2 (present)	
Body tone	91	1 (hypotonia), 2 (normal), 3 (hypertonia)	
Carriage	91	1 (normal), 2 (abnormal)	

TABLE 21.20 (continued)
Central Nervous System Function Parameters in the Beagle Dog

Observation	Number of Observations	Assessment Range	Distribution
<i>Neuromuscular</i>			
Clonic movements (home cage)	91	1 (none), 2, 3, 4, 5, 6, 7 (clonic convulsions)	
Clonic movements (open field)	91	1 (none), 2, 3, 4, 5, 6, 7 (clonic convulsions)	
Gait	91	1 (no abnormality), 2, 3, 4 (severely impaired)	
Limb position	91	1 (normal), 2 (abnormal)	
Limb tone	91	1 (hypotonia), 2 (normal), 3 (hypertonia)	
Mobility	91	1 (normal), 2, 3, 4 (severely impaired)	
Righting reflex	91	1 (normal), 2 (abnormal)	
Stereotypy	91	1 (not present), 2 (present)	
Tonic movements (home cage)	91	1 (none), 2, 3, 4, 5, 6 (severe convulsions)	
Tonic movements (open field)	91	1 (none), 2, 3, 4, 5, 6 (severe convulsions)	
<i>Physiological</i>			
Respiration	91	1 (normal), 2, 3, 4, 5 (dyspnea)	
<i>Sensorimotor</i>			
Approach response	91	1 (no response), 2 (slight response), 3, 4 (exaggerated response)	
Click response	91	1 (no response), 2 (slight response), 3, 4 (exaggerated response)	
Forelimb strength	91	1 (normal), 2, 3, 4 (severe impairment)	
Hemiwalking, left	91	1 (normal), 2, 3, 4 (severe impairment)	

(continued)

TABLE 21.20 (continued)
Central Nervous System Function Parameters in the Beagle Dog

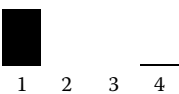
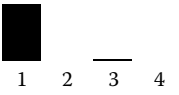

Observation	Number of Observations	Assessment Range	Distribution		
Sensorimotor					
Hemiwalking, right	91	1 (normal), 2, 3, 4 (severe impairment)			
Hindlimb strength	91	1 (normal), 2, 3, 4 (severe impairment)			
Touch response	91	1 (no response), 2 (slight response), 3, 4 (exaggerated response)			
End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Continuous Variables					
Male					
Locomotor activity count	28	62	31	1	161
Rearing	28	18	12	0	42
Defecation	28	0.5	2	0	14
Urination	28	0.1	0.48	0	3
Body temperature (°C)	28	39	0.44	38	40
Body weight (g)	28	9.9	1.7	6.9	13
Female					
Locomotor activity count	24	57	25	7	116
Rearing	24	14	9.7	0	35
Defecation	24	0.6	2.4	0	16
Urination	24	0.0	0.20	0	1
Body temperature (°C)	24	39	0.37	38	40
Body weight (g)	24	7.8	0.89	6.0	10

TABLE 21.21
Central Nervous System Function Parameters in the Nonhuman Primate
(Cynomolgus Monkey)

Observation	Number of Observations	Assessment Range	Distribution
<i>Activity/Arousal</i>			
Activity level	128	1 (very low), 2, 3 (normal), 4, 5, 6 (frenetic)	
Posture	128	1 (lying down), 2, 3 (normal), 4, 5 (frenetic)	
<i>Autonomic</i>			
Salivation	128	1 (not present), 2, 3 (excessive)	
Palpebral closure	128	1 (eyelids open), 2, 3, 4 (eyelids completely closed)	
Lacrimation	128	1 (not present), 2, 3 (excessive)	
Pupil response	128	1 (no response), 2 (normal constriction)	
<i>Neuromuscular</i>			
Tremors	128	1 (not present), 2 (present)	
Stereotypy	128	1 (not present), 2 (present)	
Fasciculation	128	1 (not present), 2 (present)	
Convulsions	128	1 (not present), 2 (present)	
Movement of facial muscles	128	1 (symmetrical), 2 (asymmetrical)	
Chaddock reflex	128	1 (negative response), 2 (positive response)	
Babinski reflex	128	1 (negative response), 2 (positive response)	
Proprioception	128	1 (not present), 2 (present)	
Paresis	128	1 (not present), 2, 3, 4, 5 (hemiparesis)	

(continued)

TABLE 21.21 (continued)
Central Nervous System Function Parameters in the Nonhuman Primate
(Cynomolgus Monkey)







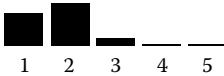

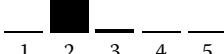
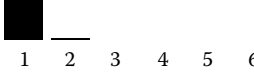



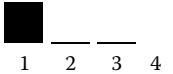
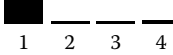

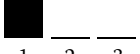


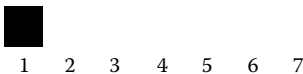

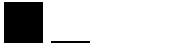


Observation	Number of Observations	Assessment Range	Distribution
<i>Neuromuscular</i>			
Ataxia	128	1 (not present), 2 (present)	 1 2
Dysmetria	128	1 (no movements), 2, 3 (not present), 4 (hypermetria)	 1 2 3 4
Slope assessment	128	1 (normal balanced movements), 2, 3 (unable to walk or climb)	 1 2 3
<i>Sensorimotor</i>			
Visual field	128	1 (follows movements symmetrically), 2, 3, 4, 5 (cannot follow movements)	 1 2 3 4 5
Auditory response	128	1 (no response), 2 (response evident)	 1 2
Response to food	128	1 (no response), 2 (smooth symmetrical movements), 3, 4 (unable to chew or swallow)	 1 2 3 4

TABLE 21.22
Central Nervous System Function Parameters in the Rat

Observation	Number of Observations	Assessment Range	Distribution
Categorical variables			
Activity/Arousal			
Ease of removal	1320	1 (very easy), 2 (easy), 3, 4, 5 (very difficult)	
General arousal	1320	1 (very depressed), 2, 3, 4 (normal), 5, 6 (very elevated)	
Handling reactivity	1320	1 (very low), 2 (low), 3, 4, 5 (high)	
Posture	1320	1 (asleep), 2, 3, 4, 5, 6 (other/abnormal)	
Vocalization	1320	1 (once), 2 (more than once)	
Autonomic			
Exophthalmus	1320	1 (not present), 2 (present)	
Lacrimation	1320	1 (none), 2, 3 (severe)	
Palpebral closure (handling)	1320	1 (eyelids wide open), 2, 3, 4 (eyelids completely shut)	
Palpebral closure (home cage)	1320	1 (eyelids wide open), 2, 3, 4 (eyelids completely shut)	
Philoerection	1320	1 (not present), 2 (present)	
Pupil response	1320	1 (normal pupil constriction), 2, 3 (lack of pupil constriction)	
Salivation	1320	1 (none), 2, 3 (severe)	
Neuromuscular			
Bizarre behavior	1320	1 (not present), 2 (present)	
Clonic movements (home cage)	1320	1 (none), 2, 3, 4, 5, 6, 7 (clonic convulsions)	
Clonic movements (open field)	1320	1 (none), 2, 3, 4, 5, 6, 7 (clonic convulsions)	
Gait	1320	1 (no abnormality), 2, 3, 4 (severely impaired)	
Mobility	1320	1 (normal), 2, 3, 4 (severely impaired)	
Righting reflex	1320	1 (normal), 2 (abnormal)	

(continued)

TABLE 21.22 (continued)
Central Nervous System Function Parameters in the Rat

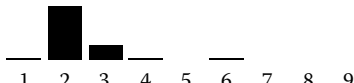
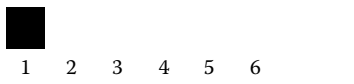
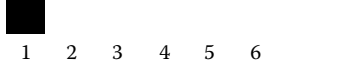
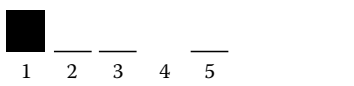
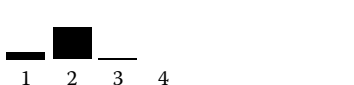
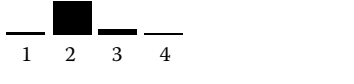
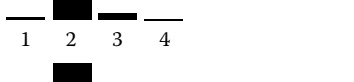
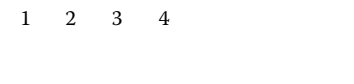



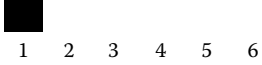



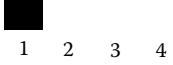
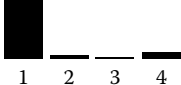




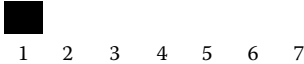
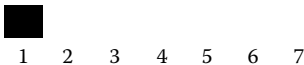
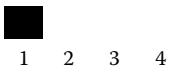
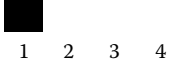

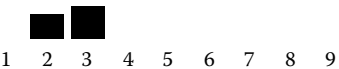
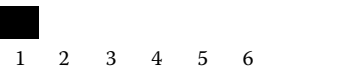
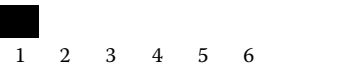
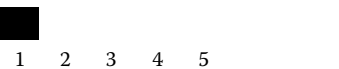


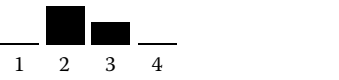

Observation	Number of Observations	Assessment Range	Distribution		
Neuromuscular					
Stereotypy	1320	1 (quiet), 2 (alert), 3, 4, 5, 6, 7, 8, 9 (self-mutilation)			
Tonic movements (home cage)	1320	1 (none), 2, 3, 4, 5, 6 (severe convulsions)			
Tonic movements (open field)	1320	1 (none), 2, 3, 4, 5, 6 (severe convulsions)			
Physiological					
Respiration	1320	1 (normal), 2, 3, 4, 5 (dyspnea)			
Sensorimotor					
Approach response	1320	1 (no response), 2 (slight response), 3, 4 (exaggerated response)			
Click response	1320	1 (no response), 2 (slight response), 3, 4 (exaggerated response)			
Tail pinch response	1290	1 (no response), 2 (slight response), 3, 4 (exaggerated response)			
Touch response	1320	1 (no response), 2 (slight response), 3, 4 (exaggerated response)			
End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
Continuous Variables					
Male					
Rearing	388	3.9	4.7	0	35
Defecation	388	1.9	2.1	0	10
Urination	388	0.70	1.20	0	12
Forelimb grip strength (kg)	388	0.28	0.13	0.033	1.141
Hindlimb grip strength (kg)	388	0.17	0.067	0.020	0.46
Hindlimb splay	378	100	17	52	160
Body temperature (°C)	388	37	0.59	34	38
Body weight (g)	388	210	34	170	350
Thermal response (s)	378	9.9	5.2	2.7	49
Female					
Rearing	296	7.9	6.5	0	34
Defecation	296	0.80	1.8	0	12
Urination	296	0.50	1.1	0	13
Forelimb grip strength (kg)	296	0.31	0.14	0.035	0.76
Hindlimb grip strength (kg)	296	0.19	0.079	0.035	0.55
Hindlimb splay	296	98	19	46	160
Body temperature (°C)	296	37	0.63	35	39
Body weight (g)	188	190	29	140	320
Thermal response (s)	296	9.7	5.6	1.1	44

TABLE 21.23
Central Nervous System Function Parameters in the Mouse (Continuous Variables)

Observation	Number of Observations	Assessment Range	Distribution
<i>Activity/Arousal</i>			
Ease of removal	100	1 (very easy), 2 (easy), 3, 4, 5 (very difficult)	
General arousal	100	1 (very depressed), 2, 3, 4 (normal), 5, 6 (very elevated)	
Handling reactivity	100	1 (very low), 2 (low), 3, 4, 5 (high)	
Posture	100	1 (asleep), 2, 3, 4, 5, 6 (other/abnormal)	
Vocalization	100	1 (once), 2 (more than once)	
<i>Autonomic</i>			
Exophthalmus	100	1 (not present), 2 (present)	
Lacrimation	100	1 (none), 2, 3 (severe)	
Palpebral closure (handling)	100	1 (eyelids wide open), 2, 3, 4 (eyelids completely shut)	
Palpebral closure (home cage)	100	1 (eyelids wide open), 2, 3, 4 (eyelids completely shut)	
Philoerection	100	1 (not present), 2 (present)	
Pupil response	100	1 (normal pupil constriction), 2, 3 (lack of pupil constriction)	
Salivation	100	1 (none), 2, 3 (severe)	
<i>Neuromuscular</i>			
Bizarre behavior	100	1 (not present), 2 (present)	
Clonic movements (home cage)	100	1 (none), 2, 3, 4, 5, 6, 7 (clonic convulsions)	
Clonic movements (open field)	100	1 (none), 2, 3, 4, 5, 6, 7 (clonic convulsions)	
Gait	100	1 (no abnormality), 2, 3, 4 (severely impaired)	
Mobility	100	1 (normal), 2, 3, 4 (severely impaired)	
Righting reflex	100	1 (normal), 2 (abnormal)	

(continued)

TABLE 21.23 (continued)
Central Nervous System Function Parameters in the Mouse (Continuous Variables)

Observation	Number of Observations	Assessment Range	Distribution
<i>Neuromuscular</i>			
Stereotypy	100	1 (quiet), 2, 3(sniffing), 4, 5, 6, 7, 8, 9 (self-mutilation)	
Tonic movements (home cage)	100	1 (none), 2, 3, 4, 5, 6 (severe convulsions)	
Tonic movements (open field)	100	1 (none), 2, 3, 4, 5, 6 (severe convulsions)	
<i>Physiological</i>			
Respiration	100	1 (normal), 2, 3, 4, 5 (dyspnea)	
<i>Sensorimotor</i>			
Approach response	100	1 (no response), 2 (slight response), 3, 4 (exaggerated response)	
Click response	100	1 (no response), 2 (slight response), 3, 4 (exaggerated response)	
Tail pinch response	100	1 (no response), 2 (slight response), 3, 4 (exaggerated response)	
Touch response	100	1 (no response), 2 (slight response), 3, 4 (exaggerated response)	

End Point	Number of Animals	Mean	Standard Deviation	Minimum	Maximum
<i>Male</i>					
Rearing	30	19	11	2	48
Defecation	30	2.4	1.9	0	7
Urination	30	0.30	0.56	0	2
Grip response (s)	30	60	2.6	36	60
Mean hindlimb splay (mm)	30	38	6.3	25	51
Body temperature (°C)	30	38	0.62	35	40
Body weight (g)	30	29	1.3	26	32
Thermal response (s)	30	22	10	4.8	51
<i>Female</i>					
Rearing	34	21	13	1	52
Defecation	34	2.6	1.8	0	8
Urination	34	0.10	0.49	0	4
Grip response (s)	34	60	2.0	42	60
Mean hindlimb splay (mm)	34	38	4.7	27	54
Body temperature (°C)	34	38	0.45	37	39
Body weight (g)	34	25	1.7	23	37
Thermal response (s)	34	21	9.1	2.7	52

CONCLUSION

Safety pharmacology studies primarily are conducted to bridge gaps in knowledge of specific functional safety concerns with new pharmaceuticals in development that would otherwise not likely be characterized adequately by the traditional toxicological evaluations executed as part of investigational programs conducted prior to the initiation of human clinical trials. The context in which this effort is realized continues to evolve with time. The current chapter has reviewed regulatory and historical factors, and current scientific and practical concerns, which have shaped contemporary practice of some essential safety pharmacology assays. Examples of current protocols, technical innovations, and associated normal behavioral and physiological reference data are included to illustrate the refinements in our definition of the discipline that must necessarily occur over time in order to best facilitate functional safety data development that will best support an increasingly diverse array of therapeutic new molecular entities.

REFERENCES

1. Anonymous. (1975). *Notes on Applications for Approval to Manufacture (Import) New Drugs*. MHW, Tokyo, Japan.
2. Anonymous. (1995). *Japanese Guidelines for Nonclinical Studies of Drugs Manual*. Yakiji Nippo Limited, Tokyo, Japan.
3. Anonymous. (1998). *Guideline for Safety Pharmacology Study*. Tokyo, Japan.
4. Anonymous. (2001). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Guidance for Industry. S7A: Safety Pharmacology Studies for Human Pharmaceuticals (ICH S7A).
5. Anonymous. (2005). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Guidance for Industry. S7B: The Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (ICH S7B).
6. Bass, A., Kinter, L., and Williams, P. (2004). Origins, practices, and future of safety pharmacology. *J. Pharmacol. Toxicol. Methods* 49:145–151.
7. Kinter, L.B. and Valentin, J.-P. (2002). Safety pharmacology and risk assessment. *Fund. Clin. Pharmacol.* 16:175–182.
8. Fung, M., Thorton, A., Mybeck, K., Hsaio-Hui Wu, J., Hornbuckle, I., and Muniz, E. (2001). Evaluation of the characteristics of safety withdrawal of prescription drugs from the worldwide pharmaceutical markets—1960 to 1999. *Drug Inform. J.* 35:293–317.
9. Schuster, D., Laggnier, C., and Langer, T. (2005). Why drugs fail—A study on the side effects in new chemical entities. *Curr. Pharm. Des.* 11:3545–3559.
10. Roden, D.M. and Anderson, M.E. (2006). Proarrhythmia. *Handbook Exp. Pharmacol.* 171:73–97.
11. Farkas, A.S. and Nattel, S. (2010). Minimizing repolarization-related proarrhythmic risk in drug development and clinical practice. *Drugs* 70:573–603.
12. Arrigoni, C. (2010). Cardiovascular liabilities of drugs: Regulatory aspects. In: Minotti, G., Ed., *Cardiotoxicity of Non-Cardiovascular Drugs*. John Wiley & Sons, West Sussex, 2010.
13. Vogel, H.G., Ed. (2002). *Drug Discovery and Evaluation: Pharmacological Assays*, Second Edition. Springer-Verlag, Berlin, Germany.
14. Ramos, K.S., Chacon, E., Acosta, D., Jr. (1996). Toxic responses of the heart and vascular systems. In: Klassen, C.D., Ed., *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Fifth Edition.
15. Fuster, V., Alexander, R.W., O'Rourke, R.A., Roberts, R., King, S.B., and Wellens, H.J.J. (2001). *Hurst's The Heart*, Tenth Edition. McGraw-Hill Medical Publishing Division, New York.
16. Anonymous. (2009). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Guidance for Industry. M3(R2): Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals. ICH M3(R2).
17. Anonymous. (2011). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Guidance for Industry. S6(R1): Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals. ICH S6(R1).
18. Anonymous. (2010). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Guidance for Industry. S9: Nonclinical Evaluation for Anticancer Pharmaceuticals (ICH S9).
19. Cavero, I. (2010). Cardiovascular system assessment best practices: A Safety Pharmacology Society meeting. *Exp. Opin. Drug Saf.* 9:855–866.
20. Leishman, D.J., Beck, T.W., Dybdal, N., Gallacher, D.J., Guth, B.D., Holbrook, M., Roche, B., and Wallis, R.M. (2011). Best practice in the conduct of key nonclinical cardiovascular safety assessments in drug development: Current recommendations from the Safety Pharmacology Society. *J. Pharmacol. Toxicol. Methods* 65:93–101.
21. Farkas, A.S. and Nattel, S. (2010). Minimizing repolarization-related proarrhythmic risk in drug development and clinical practice. *Drugs* 70:573–603.
22. Kaczorowski, G.J., Garcia, M.L., Bode, J., Hess, S.D., and Patel, U.A. (2011). The importance of being profiled: Improving drug candidate safety and efficacy using ion channel profiling. *Front. Pharmacol.* 2:1–11.
23. Polak, S., Wisniewska, B., and Brandys, J. (2009). Collation, assessment and analysis of literature *in vitro* data on hERG receptor blocking potency for subsequent modeling of drugs' cardiotoxic properties. *J. Appl. Toxicol.* 29:183–206.
24. Lee, N., Authier, S., Pugsley, M.K., and Curtis, M.J. (2010). The continuing evolution of torsades de pointes liability testing methods: Is there an end in sight? *Toxicol. Appl. Pharmacol.* 243:146–153.
25. Picard, S., Goineau, S., Guillaume, P., Henry, J., Hanouz, J.-L., and Rouet, R. (2011). Supplemental studies for cardiovascular risk assessment in safety pharmacology: A critical overview. *Cardiovasc. Toxicol.* 11:285–307.
26. Baird, T.J., Gauvin, D.V., and Dalton, J.A. (2013). Contemporary practices in core safety pharmacology assessments. In: Faqi, A.S., Ed., *A Comprehensive Guide to Toxicology in Drug Development*. Elsevier (Academic Press), London, U.K.

27. Sarazan, R.D., Mittelstadt, S., Guth, B., Koerner, J., Zhang, J., and Pettit, S. (2011). Cardiovascular function in nonclinical drug safety assessment: Current issues and opportunities. *Int. J. Toxicol.* 30:272–286.
28. Gauvin, D.V., Tilley, L.P., Smith, F.W.K., Jr., and Baird, T.J. (2006). Electrocardiogram, hemodynamics, and core body temperatures of the normal freely moving laboratory beagle dog by remote radiotelemetry. *J. Pharmacol. Toxicol. Methods* 53:128–139.
29. Gauvin, D.V., Tilley, L.P., Smith, F.W.K., Jr., and Baird, T.J. (2006). Electrocardiogram, hemodynamics, and core body temperatures of the normal freely moving cynomolgus monkey by remote radiotelemetry. *J. Pharmacol. Toxicol. Methods* 53:140–151.
30. Chiang, A.Y., Bass, A.S., Cooper, M.M., Engwall, M.J., Menton, R.G., and Thomas, K. (2007). ILSI-HESI cardiovascular safety subcommittee dataset: An analysis of the statistical properties of QT interval and rate-corrected QT interval (QTc). *J. Pharmacol. Toxicol. Methods* 56:95–102.
31. Ando, K., Hombo, T., Kanno, A., Ikeda, H., Imaizumi, M., Shimizu, N., Sakamoto, K. et al. (2005). QT PRODACT: *In vivo* QT assay with a conscious monkey for assessment of the potential for drug-induced QT interval prolongation. *J. Pharmacol. Sci.* 99:487–500.
32. Toyoshima, S., Kanno, A., Kitayama, T., Sekiya, K., Nakai, K., Haruna, M., Mino, T., Miyazaki, H., Yano, K., and Yamamoto, K. (2005). QT PRODACT: *In vivo* QT assay in the conscious dog for assessing the potential for QT interval prolongation by human pharmaceuticals. *J. Pharmacol. Sci.* 99:459–471.
33. Murphy, D.J. (2003). Respiratory function assessment in safety pharmacology. *Curr. Protocols Pharmacol.* 10.9.1–10.9.11.
34. Parent, R.A. (1992). *Treatise on Pulmonary Toxicology: Comparative Biology of the Normal Lung*, Vol. 1. CRC Press, Boca Raton, FL.
35. Levitzky, M.G. (2003). *Pulmonary Physiology*, Sixth Edition. McGraw Hill, New York.
36. Hoymann, H.G. (2007). Invasive and noninvasive lung function measurements in rodents. *J. Pharmacol. Toxicol. Methods* 55:16–26.
37. Authier, S., Haefner, P., Fournier, S., Troncy, E., and Moon, B. (2010). Combined cardiopulmonary assessments with implantable telemetry device in conscious freely moving cynomolgus monkeys. *J. Pharmacol. Toxicol. Methods* 62:6–11.
38. Ingram-Ross, J.L., Curran, A.K., Miyamoto, M., Sheehan, J., Thomas, G., Verbeeck, J., deWaal, E.J., Verstynen, B., and Pugsley, M.K. (2012). Cardiorespiratory safety evaluation in non-human primates. *J. Pharmacol. Toxicol. Methods* 66(2):114–124.
39. Schierok, H., Markert, M., Pairet, M., and Guth, B. (2000). Continuous assessment of multiple vital physiological functions in conscious freely moving rats using telemetry and a plethysmography system. *J. Pharmacol. Toxicol. Methods* 43:221–217.
40. Delaunois, A., Dedoncker, P., Hanon, E., and Guyaux, M. (2009). Repeated assessment of cardiovascular and respiratory functions using combined telemetry and whole-body plethysmography in the rat. *J. Pharmacol. Toxicol. Methods* 60:117–129.
41. Moser, V.C., McCormick, J.P., Creason, J.P., and MacPhail, R.C. (1988). Comparison of chlordimeform and carbaryl using a functional observational battery. *Fund. Appl. Toxicol.* 11:189–206.
42. Lindgren, S., Bass, A.S., Briscoe, R., Burse, K., Friedrich, G.S., Kallman, M.J., Markgraf, C., Patmore, L., and Pugsley, M.K. (2008). Benchmarking safety pharmacology regulatory packages and best practice. *J. Pharmacol. Toxicol. Methods* 58:99–109.
43. Redfern, W.S., Strang, I., Storey, S., Heys, C., Barnard, C., Lawton, K., Hammond, T.G., and Valentin, J.-P. (2005). Spectrum of effects detected in the rat functional observational battery following oral administration of non-CNS targeted compounds. *J. Pharmacol. Toxicol. Methods* 52:77–82.
44. Markgraf, C.G., Cirino, M., and Meredith, J. (2010). Comparison of methods for analysis of functional observational battery (FOB) data. *J. Pharmacol. Toxicol. Methods* 62:89–94.
45. Haggerty, G.C. (1991). Strategy for and experience with neurotoxicity testing of new pharmaceuticals. *Intern. J. Toxicol.* 10:677–688.
46. Wasielewski, J.A., White, J.C., Newton, P.E., Briscoe, R.J., and Baird, T.J. (2002). Development and validation of a canine functional observational battery (FOB) for use in toxicity studies. *J. Soc. Toxicol.* 66(1eS):1286.
47. Gauvin, D.V. and Baird, T.J. (2008). A functional observational battery in non-human primates for regulatory-required neurobehavioral assessments. *J. Pharmacol. Toxicol. Methods* 58:88–93.
48. Guth, B.D., Bass, A.S., Briscoe, R., Chivers, S., Markert, M., Siegl, P.K.S., and Valentin, J.-P. (2009). Comparison of electrocardiographic analysis for risk of QT interval prolongation using safety pharmacology and toxicological studies. *J. Pharmacol. Toxicol. Methods* 60:107–116.
49. Gad, S.C. (2004). *Safety Pharmacology in Pharmaceutical Development and Approval*. CRC Press, LLC, Boca Raton, FL.
50. Cavero, I. (2009). Exploratory safety pharmacology: A new safety paradigm to de-risk drug candidates prior to selection for regulatory science investigations. *Exp. Opin. Drug Saf.* 8:627–647.
51. Baldrick, P. (2008). Safety evaluation to support First-In-Man investigations I: Kinetic and safety pharmacology studies. *Regul. Toxicol. Pharmacol.* 51:230–236.
52. Bernton, E.W. (2008). Safety pharmacology: Similarities and differences between small molecules and novel biopharmaceuticals. In: Cavagnaro, J.A., Ed., *Preclinical Safety Evaluation of Biopharmaceuticals: A Science-Based Approach to Facilitating Clinical Trials*. John Wiley & Sons, Hoboken, NJ, 2008.

22 Risk Assessment

Michael J. Derelanko, PhD, DABT, FATS

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INTRODUCTION

The purpose of this chapter is to provide information to familiarize the reader with the basic concepts and process of risk assessment and to provide a handy and quick reference source for standard assumptions, terminology, equations, and values used in performing these assessments. Although the information presented in this chapter is generally representative of the risk assessment process as currently practiced by regulatory bodies such as the US Environmental Protection Agency, risk assessment is an ever-evolving discipline. As such, methods and procedures are often modified or replaced to reflect current scientific practice and theory.

There is often little or no scientific evidence to support many of the concepts and assumptions on which the risk assessment process is based. Therefore, other approaches for assessing risk may be equally valid. Moreover, the likelihood that all individuals in all situations can fit into any standardized model is extremely remote. In practice, each risk assessment should ideally be unique and the use of standardized assumptions and values may not always be appropriate.

Risk assessment is an imprecise science. Many assumptions are made where actual data are not available or difficult to obtain. Many of the models are based on hypothetical mechanisms of toxicity, which may not be scientifically valid. Because of these limitations, a great deal of uncertainty is inherent in the risk assessment process. Due to this uncertainty and the desire to protect human populations under a variety of exposure scenarios, risk assessments generally are conservative and overestimate risk.

This chapter is divided into seven sections. “Risk Assessment: Introduction and Overview” section presents an overview of the risk assessment process and outlines some of the basic theoretical considerations and concepts that form the foundation on which the risk assessment process is based.

“Dose–Response Relationships” section deals with dose–response relationships and their use in risk assessment.

“Epidemiology” section covers basic terminology, methodology, and concepts of epidemiology. In addition, reference information is presented on causation and incidence of human cancer and reproductive and developmental effects. “Relative Risk Tables” section contains relative risk tables useful for making risk comparisons. Standard reference values for use in interspecies extrapolation and exposure assessment are presented in “Standard Risk Assessment Reference Values” section. “Physiologically Based Pharmacokinetic Modeling” section provides a brief overview of physiologically based pharmacokinetic (PBPK) modeling and presents reference values for physiological and biochemical parameters used in these models. Equations for calculations commonly encountered in risk assessments can be found in “Risk Assessment Calculations” section. Toxicity classification schemes for chemicals previously provided in this chapter in the second edition of the *Handbook of Toxicology* can now be found in Chapter 19 (Chemical Toxicology). A glossary containing common risk assessment terms, acronyms, and abbreviations can be found at the end of the book.

RISK ASSESSMENT: INTRODUCTION AND OVERVIEW

With few exceptions such as veterinary and agricultural products, toxicology studies are not conducted solely to assess the toxic effects of chemicals in animals but to identify the effects that might occur in humans. Risk assessment is the process of evaluating the toxic properties of chemicals and the conditions of human exposure to ascertain the likelihood that humans will be adversely affected and to characterize the nature of the effects, which may be experienced.

The risk assessment process, such as performed by the US Environmental Protection Agency, can be divided into four steps: hazard identification, dose–response assessment, exposure assessment, and risk characterization (Figure 22.1). In hazard

identification, a determination is made of whether a substance of concern, be it a pharmaceutical, industrial chemical, environmental pollutant, etc., can be linked to an adverse effect. Dose–response assessment establishes relationships between the magnitude of exposure and the occurrence of the adverse effect. The major activities of toxicologists are concentrated in these two steps. In exposure assessment, human exposure to the substance of concern is identified through characterization of the exposed population, routes of exposure, and magnitude of the exposure under various conditions. All the information derived in these three steps of the risk assessment process is used in the risk characterization step. In this fourth and final stage of the risk assessment process, a determination is made of the likelihood that humans may experience the identified adverse effect under actual or plausible hypothetical conditions of exposure.

Based on the risk characterization, the need for and the degree of risk management will be determined. A number of

options are available to the risk manager, including education and communication of risk, exposure monitoring and controls, limitations in the use of the substance of concern, or a total ban of the chemical. Risk management decisions are influenced by economic, political, and social concerns. Risk management is considered separately from the risk assessment process. Further discussion of risk management is beyond the scope of this book.

An overview of some of the major concepts that form the foundation of the risk assessment process, as well as assumptions and factors considered and/or used in risk assessment, is presented in the tables and figures of this section. A review of this information will provide a basic understanding of risk assessment (Figures 22.2 through 22.10; Tables 22.1 through 22.7). The reader should consult the cited references for more detailed information on the risk assessment process.

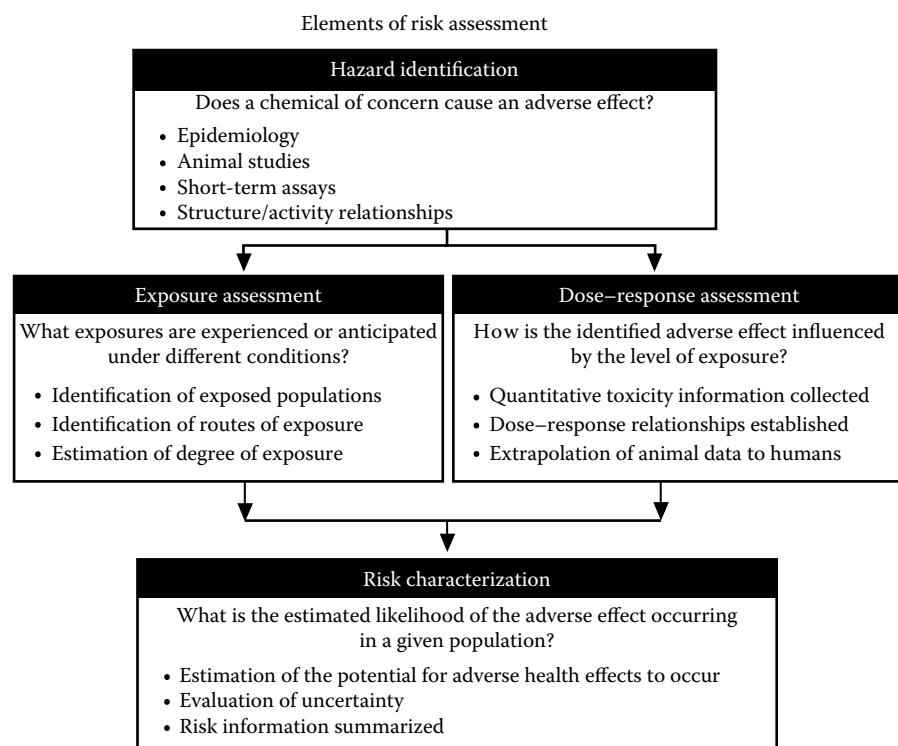


FIGURE 22.1 The four major elements of risk assessment. *Hazard identification*—in this step, a determination is made of whether a chemical of concern is or is not causally linked to a particular health effect. Information can come from human and animal studies, *in vitro* assays, and through analogy to structurally similar chemicals. *Exposure assessment*—involves the characterization of the amount, frequency, and duration of human exposure. Determinations are made of the concentration of hazardous substances in media (i.e., air, water, soil), magnitude and pathways of exposure, environmental fate, and populations at risk. The purpose of this step is to provide a quantitative estimate of human exposure. *Dose–response assessment*—the relationship between the magnitude of exposure and the occurrence of the expected health effects is determined in this step. Information obtained from animal studies is extrapolated to humans. Generally, different assessments are performed for noncarcinogenic and carcinogenic materials. Along with hazard identification, the major activities of most toxicologists are focused on this portion of the risk assessment process. *Risk characterization*—in this final stage of the risk assessment process, information from the three previous steps are evaluated to produce a determination of the nature and magnitude of human risk. The risk assessment process is completed with a summary of the risk information. The information developed in the risk assessment process will be utilized in the *risk management process* in which decisions are made as to the need for, the degree of, and the steps to be taken to control exposures to the chemical of concern. (From U.S. Environmental Protection Agency, *General Quantitative Risk Assessment Guidance for Non-Cancer Health Effects*, ECAP-CIN-538M, 1989; National Research Council, *Risk Assessment in the Federal Government*, National Academy Press, Washington, DC, 1983; Adapted from Hooper, L.D. et al., Risk assessment for toxic hazards, Chapter 7, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992.)

TABLE 22.1
Hierarchy Data Selection for Risk Assessment

1. Human data *lifetime exposure* via the more appropriate route (inhalation or oral)
2. Human data *less-than-lifetime exposure* with lifetime observation (exposure via the more appropriate route)
3. Human data *less-than-lifetime exposure* with less-than-lifetime observation (exposure via the more appropriate route)
4. Human data *lifetime exposure* via the less appropriate route if reasonable toxicologically
5. Human data *less-than-lifetime exposure* with lifetime observation (exposure via the less appropriate route)
6. Human data *less-than-lifetime exposure* with less-than-lifetime observation (exposure via the less appropriate route)
7. Animal data the same sequence as for human data. Animal studies of less than 90 days of exposure and/or less than 18 months of observation from first exposure should not be used

Source: Adapted from Hallenbeck, W.H. and Cunningham, K.M., Qualitative evaluation of human and animal studies, Chapter 3, in *Quantitative Risk Assessment for Environmental and Occupational Health*, Lewis Publishers, Chelsea, MI, 1986. With permission.

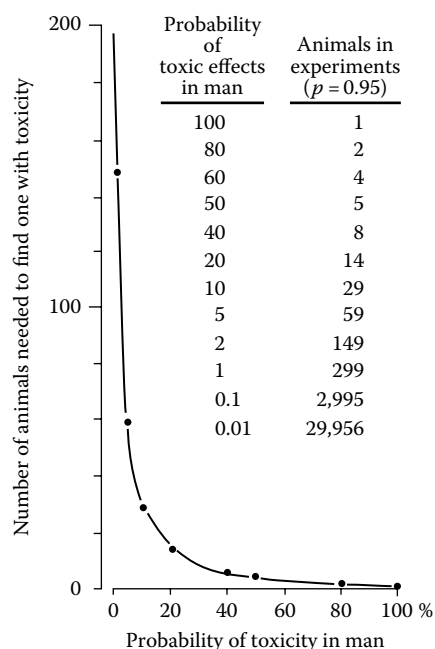


FIGURE 22.2 The interrelationship between the probability (percent) of a given toxic effect in humans and the number of animals required in a toxicology study to ensure that the same toxic effect can be observed. With a high incidence of occurrence in humans, few animals are needed, but because the incidence frequently is very low, then astronomical numbers of animals would be required to “guarantee” that the same effect would be observed. This relationship is usually cited as the reason for using unrealistically large dose levels in animal studies to increase the probability that toxic effects will be observed with relatively small numbers of animals. (Data derived from Zbinden, G., *Progress in Toxicology*, Vol. 1, Springer-Verlag, New York, 1973; Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 2. With permission.)

TABLE 22.2
Typical Factors Considered in a Risk Assessment

Physical and chemical properties of the chemical
 Patterns of use
 Handling procedures
 Availability and reliability of control measures
 Source and route of exposure under ordinary and extraordinary conditions
 Potential for misuse
 Magnitude, duration, and frequency of exposure
 Nature of exposure (oral, dermal, inhalation)
 Physical nature of the exposure (solid, liquid, vapor, etc.)
 Influence of environmental conditions of exposure
 Population exposed
 Number
 Sex
 Health status
 Personal habits (e.g., smoking)
 Lifestyles (e.g., hobbies, activities)

Source: Ballantyne, B. and Sullivan, J.B., Basic principles of toxicology, Chapter 2, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.

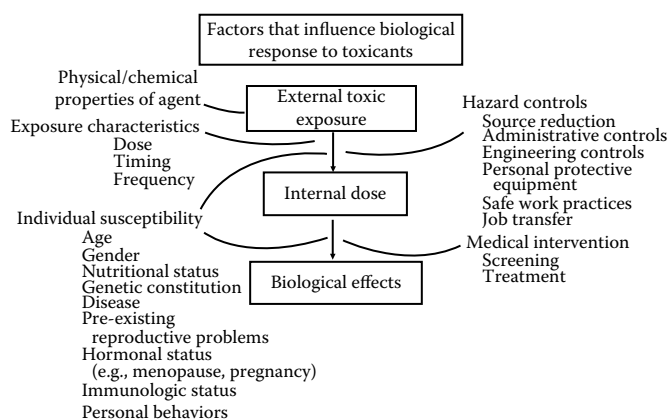


FIGURE 22.3 Factors that influence the risk posed to an individual by xenobiotic exposures. These factors as well as others should be considered in a well-conducted risk assessment. Historically, risk assessments have been based on external exposures, which create large uncertainties in extrapolating animal data to humans. The use of internal dose can provide more accurate interspecies extrapolation resulting in a more realistic characterization of risk. (From Paul, M., Clinical evaluation and management, Chapter 10, in *Occupational and Environmental Reproductive Hazards: A Guide for Clinicians*, Williams & Wilkins, Baltimore, MD, 1993. With permission.)

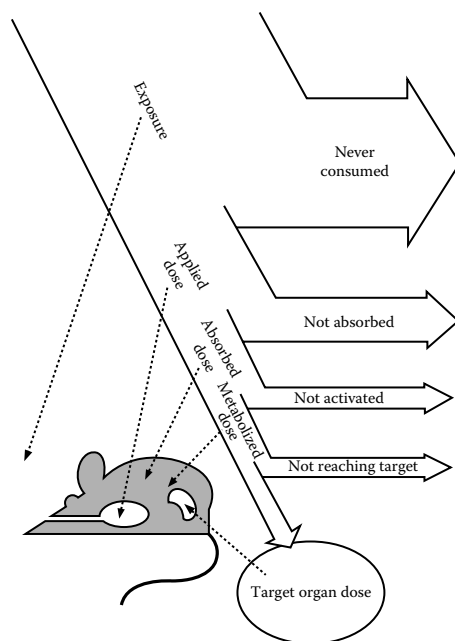


FIGURE 22.4 Representation of the relationships between ambient exposure and critical target dose and the progressive decrease in effective exposure due to various biological barriers. (From *Low-Dose Extrapolation of Cancer Risks: Issues and Perspectives*, International Life Sciences Institute, Washington, DC, p. 188, Copyright 1995. With permission.)

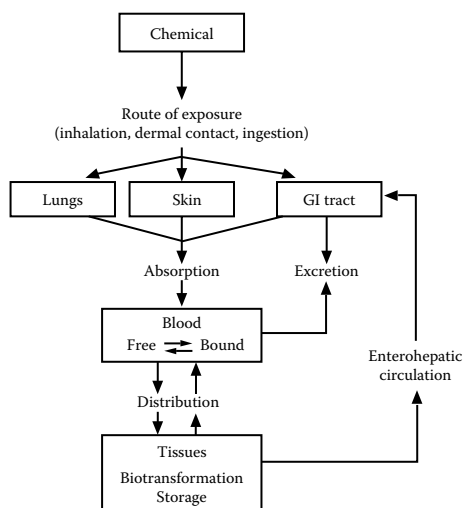


FIGURE 22.5 Diagrammatic representation of the possible pharmacokinetic fate of a chemical after exposure by inhalation, dermal contact, and ingestion. The lungs and skin also have enzyme systems capable of biotransformation (not shown). The fate of a chemical after exposure can vary considerably between species. Pharmacokinetic information is essential for accurate risk assessments. Such information can be obtained from animal studies or PBPK models ("Physiologically Based Pharmacokinetic Modeling" section). In the absence of such data, assumptions are often made that introduce a great degree of uncertainty into the risk assessment process. (Modified from Ballantyne, B. and Sullivan, J.B., Basic principles of toxicology, Chapter 2, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992.)

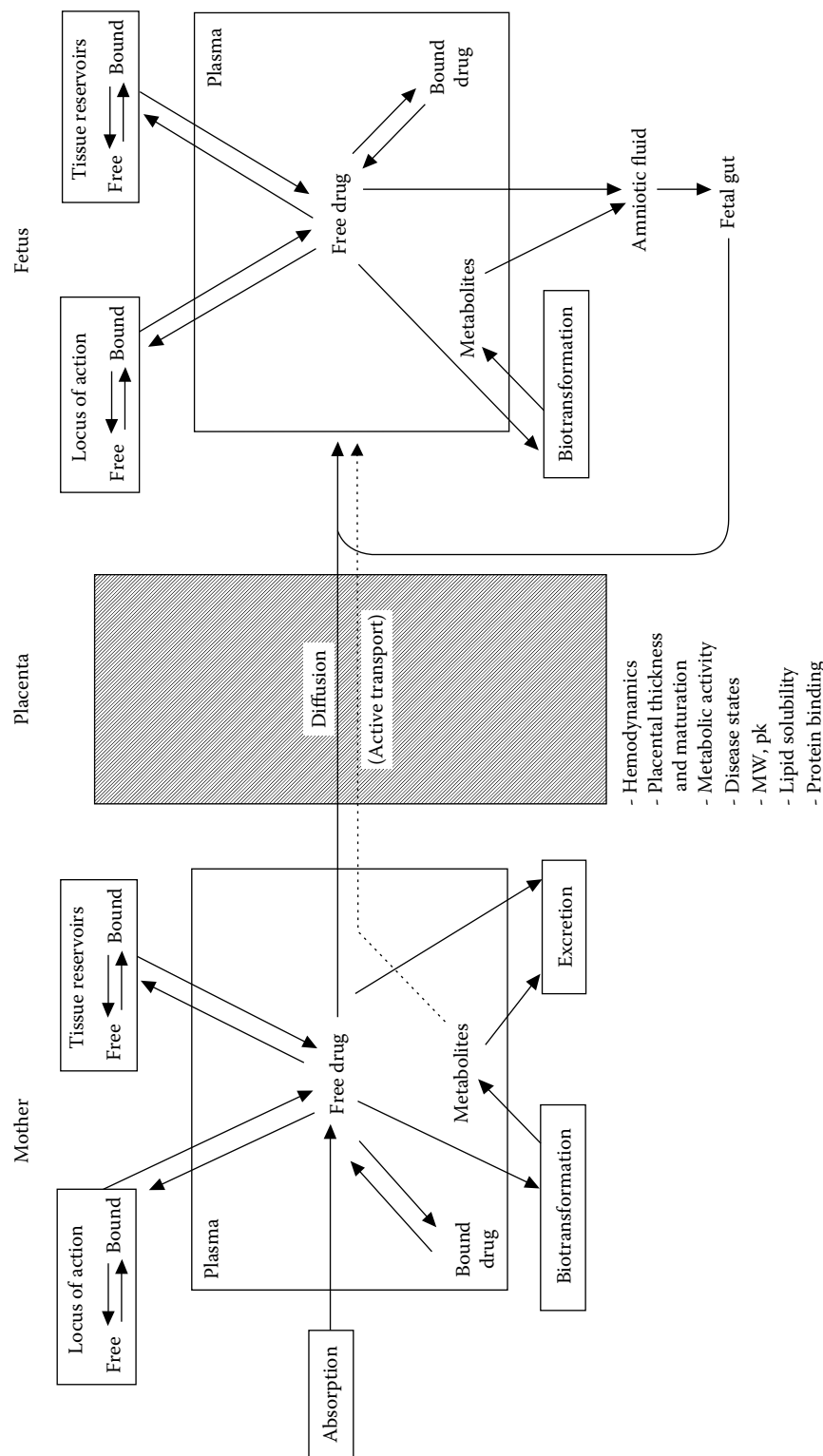


FIGURE 22.6 Maternal–placental–fetal pharmacokinetic relationships that can influence the risk posed by a reproductive toxin. (Adapted from Chow, A.W. and Jewesson, P.J., *Rev. Infect. Dis.*, 7, 288, 1985; McGuigan, M.A., Teratogenesis and reproductive toxicology, Chapter 16, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1993. With permission.)

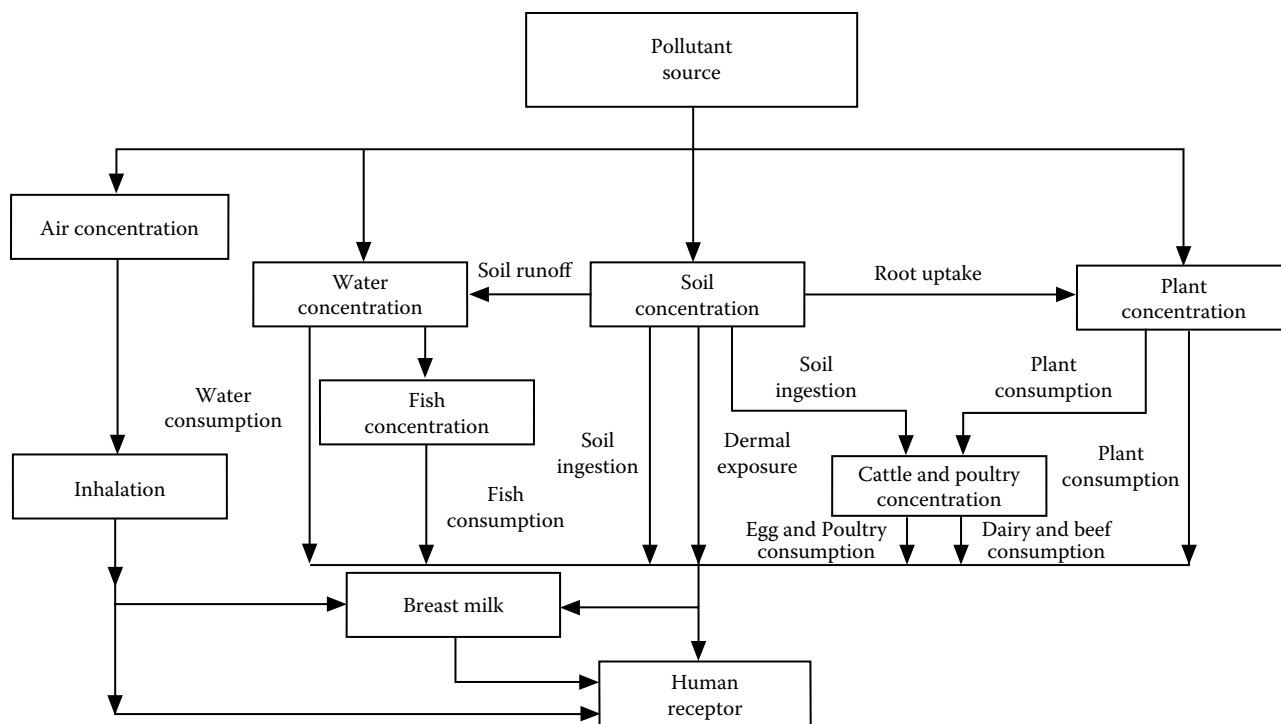


FIGURE 22.7 Diagrammatic representation of potential exposure pathways available to an environmental pollutant. Limitations in analytical methodology (e.g., sampling, analytical sensitivity, accuracy) used in measuring concentrations of chemicals in media (i.e., air, water) can introduce uncertainty into a risk assessment. Modeled data are often used in the absence of actual analytical measurements. Exposure estimates are generally based on standard exposure values (i.e., water consumption, soil ingestion), which can range from average to worst case (see Table 22.43). In general, the accuracy of an exposure assessment and subsequent risk characterization is only as good as the information from which it was made. (Modified from Lowe, J.A. et al., *Health Effects of Municipal Waste Incineration*, CRC Press, Boca Raton, FL, 1990.)

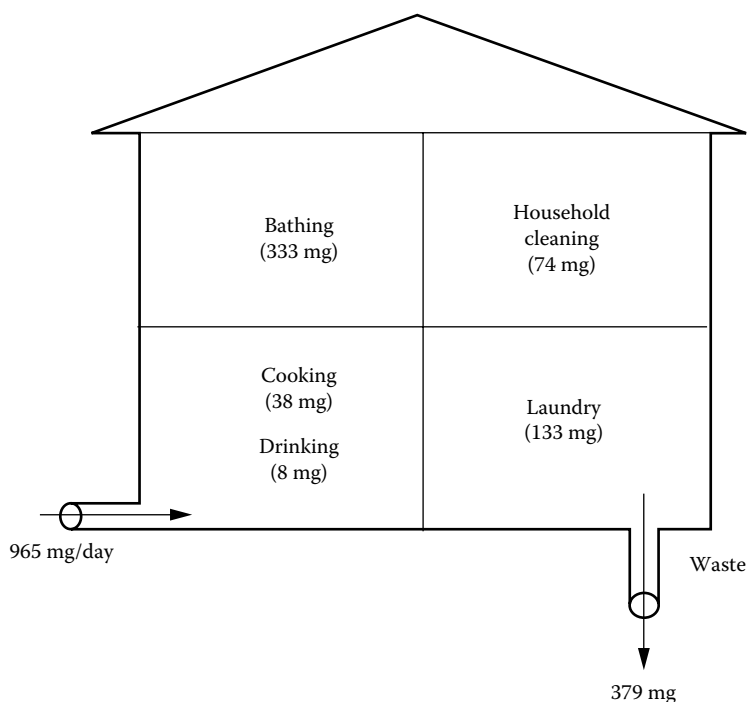


FIGURE 22.8 Example of the distribution of a contaminant present in household water at a concentration of 1 mg/L based on household water use patterns. Presented as an example of the type of data generated in an exposure assessment. Such information is necessary to develop estimates of exposure (see Table 22.3). (Adapted from U.S. Environmental Protection Agency, *Techniques for the Assessment of the Carcinogenic Risk to the U.S. Population due to Exposure from Selected Volatile Organic Compounds from Drinking Water via the Ingestion, Inhalation and Dermal Routes*, Cothorn, C.R., Coniglio, W.A., and Marcus, W.L. Eds., Office of Drinking Water, NTIS, PB 84-213941, 1984.)

TABLE 22.3
Comparative Assessment of Absorbed Dose from a Volatile Pollutant
(100 µg/L) in Drinking Water

	Absorbed Dose (µg/Day)				
	Formula-Fed Infant (4 kg)	Preteen (32 kg)	Adult Female (60 kg)	Adult Male ^a (70 kg)	Male ^b (70 kg)
Fluid ingestion	80	150	200	200	200
Inhalation of enriched indoor air	10	10	70	50	50
Inhalation of enclosed shower air	—	100	300	200	—
Dermal absorption bathing	0.02–0.06	—	—	—	2.5
Swimming	—	10–300	—	—	—
Total absorbed dose, µg/kg/day	20	10–20	10	7	4

Source: U.S. Environmental Protection Agency, *Techniques for the Assessment of the Carcinogenic Risk to the U.S. Population due to Exposure from Selected Volatile Organic Compounds from Drinking Water via the Ingestion, Inhalation and Dermal Routes*, Cothorn, C.R., Coniglio, W.A., and Marcus, W.L. Eds., Office of Drinking Water, NTIS, PB 84-213941, 1984.

^a Showering adult male.

^b Adult male that does not take showers.

TABLE 22.4
Typical Assumptions Made in a Risk Assessment of an Animal
Carcinogen Water Pollutant

National monitoring is representative of the existing exposure profile
 The equivalent amount of toxicant per liter in water is transferred to a liter of air
 100% of the toxicant in water is released to the air
 Everyone is exposed to the same level of the contaminant
 The average human ingests 2 L of drinking water per day and daily inhales an average of 20 m³ of air
 All of the ingested or inhaled toxicant is absorbed into the blood
 Dermal exposure is insignificant compared with oral and inhalation exposures
 The same effects observed in animals will occur in humans
 The only difference between humans and animals is one of scale
 There are no thresholds
 Both benign and malignant tumors are indicative of cancer
 The mathematical expression used to reflect the biological dose response is curvilinear at low doses

Source: U.S. Environmental Protection Agency, *Techniques for the Assessment of the Carcinogenic Risk to the U.S. Population due to Exposure from Selected Volatile Organic Compounds from Drinking Water via the Ingestion, Inhalation and Dermal Routes*, Cothorn, C.R., Coniglio, W.A., and Marcus, W.L. Eds., Office of Drinking Water, NTIS, PB 84-213941, 1984.

TABLE 22.5
Major Factors That Influence a Risk Assessment

Factor	Effect
Low-dose extrapolation	Can involve as many as 50 or more assumptions each of which introduce uncertainty Often considered the greatest weakness in risk assessment
Population variation	The use of standard exposure factors can underestimate actual risk to hypersensitive individuals. Addressing the risk assessment to the most sensitive individuals can overestimate risk to the population as a whole
Exposure variation	The use of modeling and measurement techniques can provide exposure estimates that diverge widely from reality
Environmental variation	Can affect actual exposures to a greater or lesser degree than assumed to exist
Multiple exposures	Risk assessments generally deal with one contaminant for which additive, synergistic, and antagonistic effects are unaccounted. Can result in underestimate or overestimate of risk
Species differences	It is generally assumed that humans are equivalent to the most sensitive species. Can overestimate or underestimate risk
Dose based on body weight	Toxicity generally does not vary linearly with body weight but exponentially with body surface area
Choice of dose levels	Use of unrealistically high-dose levels can result in toxicity unlikely to occur at actual exposure levels. The number of animals being studied may be insufficient to detect toxicity at lower doses
UFs	The use of UFs in attempting to counter the potential uncertainty of a risk assessment can overestimate risk by several orders of magnitude
Confidence intervals	The upper confidence interval does not represent the true likelihood of an event and can overestimate risk by an order of magnitude or more
Statistics	Experimental data may be inadequate for statistical analysis. Statistical significance may not indicate biological significance, and a biologically significant effect may not be statistically significant. Statistical significance does not prove causality. Conversely, lack of statistical significance does not prove safety

TABLE 22.6
Criteria Defining "High-Exposure" Chemicals

Production greater than 100,000 kg
More than 1000 workers exposed
More than 100 workers exposed by inhalation to greater than 10 mg/kg/day
More than 100 workers exposed by inhalation to 1–10 mg/day for more than 100 days/year
More than 250 workers exposed by routine dermal contact for more than 100 days/year
Presence of the chemical in any consumer product in which the physical state of the chemical in the product and the manner of use would make exposure likely
More than 70 mg/year of exposure via surface water
More than 70 mg/year of exposure via air
More than 70 mg/year of exposure via groundwater
More than 10,000 kg/year release to environmental media
More than 1000 kg/year total release to surface water after calculated estimates of treatment

Source: U.S. Environmental Protection Agency, *Reported in Pesticide and Toxic Chemical News*, October 19, p. 34, 1988.

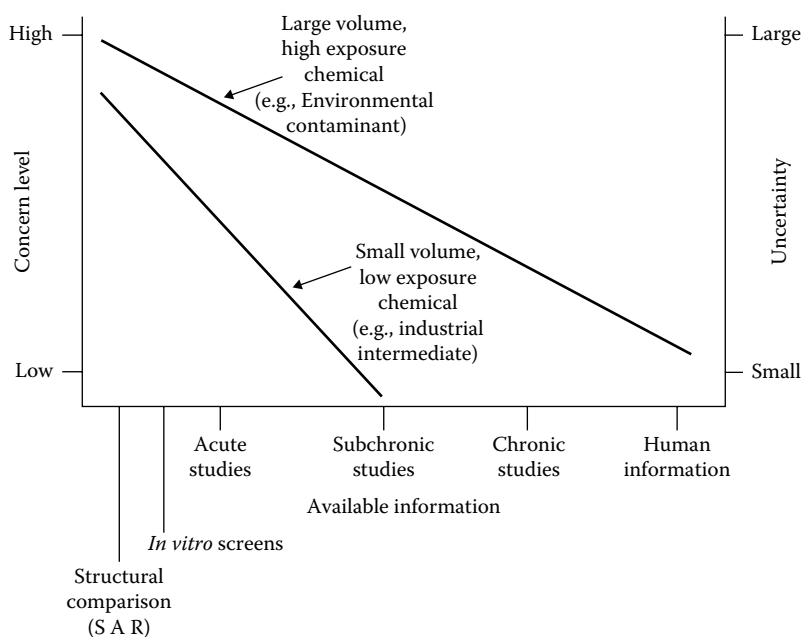


FIGURE 22.9 Relationship between the degree of uncertainty associated with the risk assessment of a chemical, the concern for human exposure, and the toxicological information available on the chemical. In practice, the larger the toxicological database available on a chemical of concern (“weight-of-evidence”), the greater the certainty (less uncertainty) that the estimated “safe” exposure level will be protective of individuals exposed to the chemical. Similarly, the concern that the risk assessment will underestimate the risk decreases with a larger toxicological database. Generally, less toxicological information will be required to reduce the concern level and uncertainty associated with a small-volume, low-exposure chemical (for which the exposed population is well characterized and the exposures can be controlled) as compared with a large-volume, high-exposure chemical.

TABLE 22.7

Factors That Influence Risk Management Decisions

Decreases Degree of Risk Management	Increases Degree of Risk Management
Risk assumed voluntarily	Risk borne involuntarily
No alternatives available	Safer alternatives available
Exposure is essential	Exposure considered a luxury
Exposure primarily occupational	Exposure nonoccupational
Exposure primarily to average individuals	Exposure involves hypersensitive individuals
Intended use can be reasonably guaranteed	Potential for misuse high
Toxic effects reversible	Toxic effects permanent
Toxic effects not inheritable	Toxic effects inheritable
Risk perceived acceptable	Risk perceived unacceptable

Source: Adapted from Lowrance, W.M., *Of Acceptable Risk: Science and the Determination of Safety*, William Kaufmann, Los Altos, CA, 1976, Chapter 3.

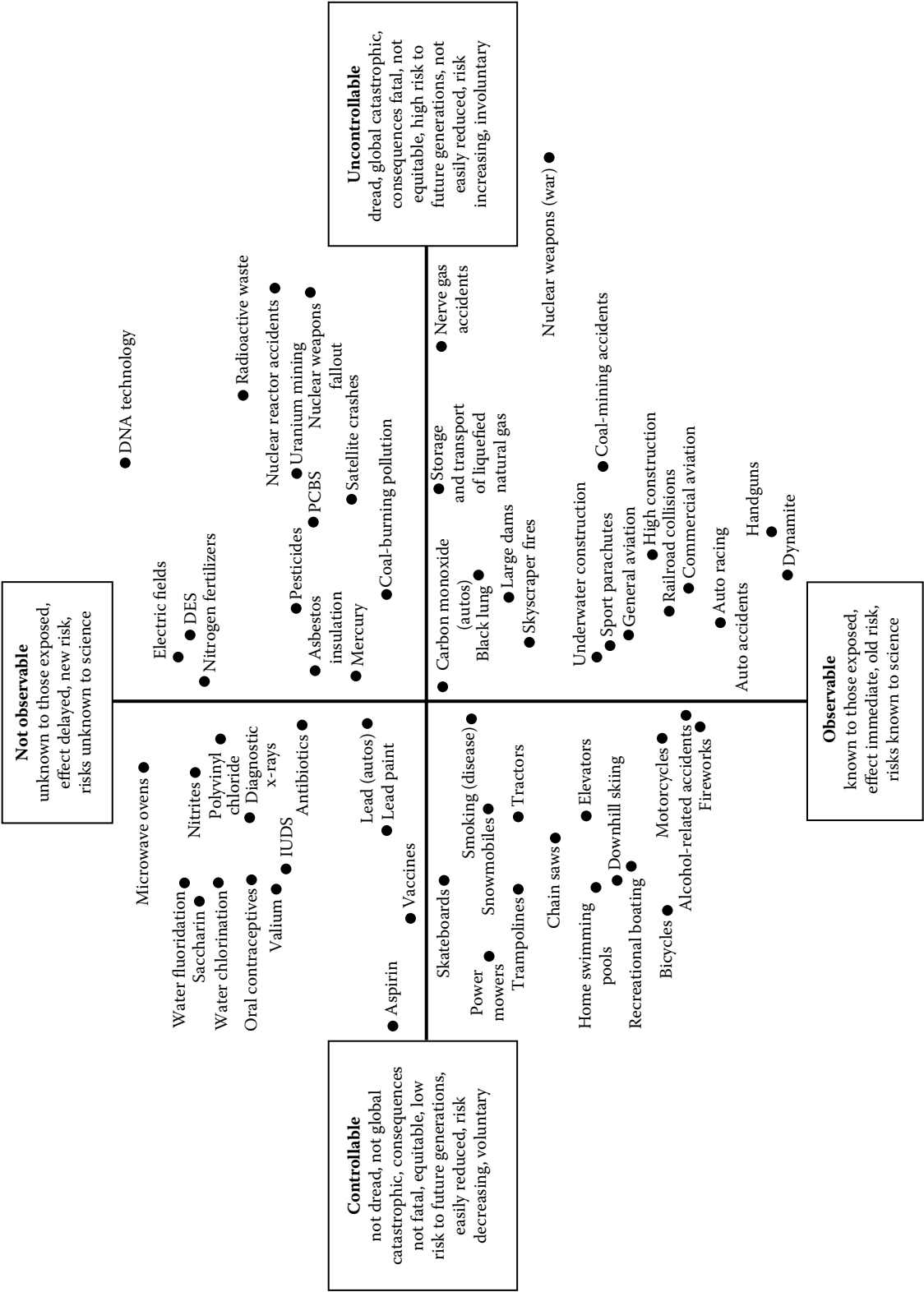


FIGURE 22.10 Graphic representation of risk perception that roughly corresponds to a hazard's "dreadfulness" and degree to which it is understood. Public concern increases above the horizontal axis and to the right of the vertical axis. Conversely, concern decreases below the horizontal axis and left of the vertical axis. Factors that impact on the perception of risk influence risk management decisions. (Illustration by J. Johnson; From Morgan, M.G., *Scientific Am.*, 269, 32, Copyright 1993. All rights reserved.)

DOSE-RESPONSE RELATIONSHIPS (FIGURES 22.11 THROUGH 22.27; TABLE 22.8)

The dose-response relationship forms the basis of the most fundamental concept of toxicology, that the toxicity of any material is defined by its dose-response curve or, to paraphrase Paracelsus, “the dose makes the poison.” Dose-response assessment is one of the four steps in the risk assessment process (see Figure 22.1).

The dose-response curve generally takes two forms: the first displays the distribution of an effect within a population as a function of changing exposure, and the second indicates the degree of change of an effect in an exposed individual of a population as a function of changing exposure. The demonstration of dose-response suggests causality.

The typical dose-response curve is sigmoidal but can also be linear, concave, convex, or bimodal. The shape of the curve can offer clues to the mechanism of action of the toxin, indicate multiple toxic effects, and identify the existence and extent of sensitive subpopulations. Analysis of the dose-response curve can demonstrate average response, the degree of susceptibility within a population, and the range of exposure affecting hyperreactive individuals. The slope of the dose-response curve categorizes the potency of the toxin and indicates the magnitude of effect associated with incremental increases in exposure. The curve can display the degree of confidence (and conversely uncertainty) associated with the data. The reader should refer to general toxicology and pharmacology textbooks for a more detailed discussion of dose-response relationships.

The purpose of this section is to provide examples of some of the ways dose-response relationships are used

in risk assessment and the type of information that can be obtained from the dose-response curve. Risk assessment takes advantage of the ability of the dose-response curve to quantitate the susceptibility of individuals in an exposed population to a substance of concern. Dose-response assessment determines the relationship between the extent (magnitude) of exposure and the probability of occurrence of health effects. For pharmaceuticals, safety ratios such as the therapeutic index and margin of safety are derived. “Safe levels” of exposure such as reference dose (RfD), acceptable daily intake (ADI), and permissible exposure limits (PELs) for pollutants, food additives, and industrial chemicals, respectively, are estimated from dose-response.

The previous values are determined for substances demonstrated to have a threshold below that an effect of concern is not expected to occur. Where thresholds are assumed not to exist, such as for carcinogens, virtually safe doses (VSDs) or exposures are determined by extrapolation of the dose-response curve to levels of risk deemed acceptable by society. The reliability of this approach is compromised by the uncertainty associated with modeling the low-dose region of the dose-response curve.

The quality of a dose-response assessment is only as good as the data with which the dose-response curve was generated. Dose-response assessment, coupled with exposure assessment, allows the risk of exposure to a chemical to be characterized, which ultimately is the purpose of the risk assessment process. The more accurate the dose-response and exposure assessment, the more realistic the risk characterization will be.

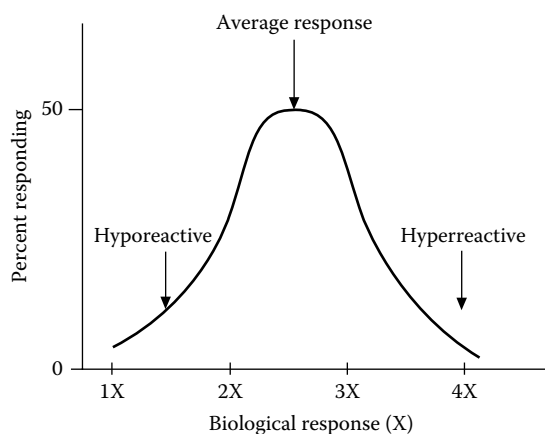


FIGURE 22.11 Typical frequency distribution of a population response to an equivalent dose of biologically active agent. This type of response represents the variability that occurs within biological systems and is the basis for the concept of dose response in pharmacology and toxicology. This figure demonstrates that within any population, both hyporeactive and hyperreactive individuals can be expected to exist and must be addressed in a risk assessment.

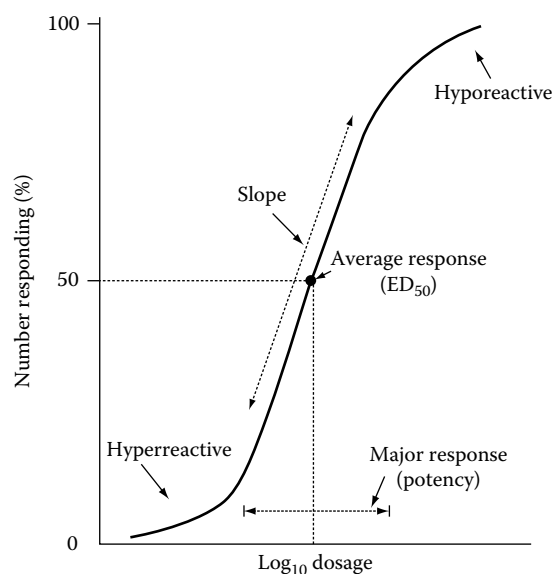


FIGURE 22.12 Typical sigmoid cumulative dose-response curve for a toxic effect that is symmetrical about the average (50% response) point. Note dosage is presented on a log scale. The major response (potency) occurs around the average response. The midpoint of the curve is referred to as the median effective dose (ED_{50}) for the effect being considered. If mortality is the end point, this point is referred to as the median lethal dose (LD_{50}). The 95% confidence limits are the narrowest at the midpoint (see Figure 22.14), which makes this the point most useful for comparison of toxicity between chemicals. The slope of the curve is determined by the increase in response as a function of incremental increases in dosage. A steep slope indicates a majority of a population will respond within a narrow dose range, while a flatter curve indicates that a much wider dose range is required to affect a majority of the exposed population (see Figure 22.15). Hyperreactive and hyporeactive individuals are at the extreme left and right sides of the curve, respectively. (From Ballantyne, B., *Exposure-dose-response relationships*, Chapter 3, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.)

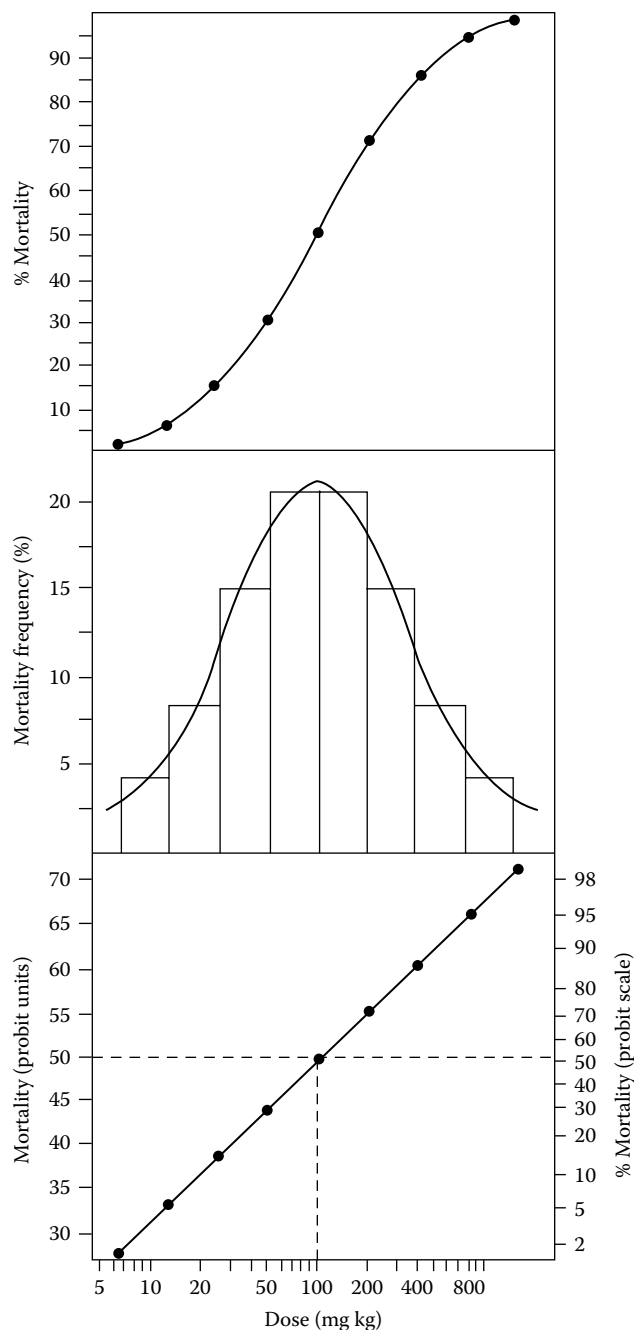


FIGURE 22.13 Various ways of presenting dose-response data. The abscissa is a log dosage of the chemical. The ordinate of the top panel is percent mortality producing a sigmoidal curve (see Figure 22.12); a bell-shaped curve is produced when the ordinate is mortality frequency (middle panel); the response is linearized when the ordinate is in probit units (bottom panel). (From Klaassen, C.D. and Eaton, D.L., *Principles of toxicology*, Chapter 2, in *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th edn., Doull, J., Klaassen, C.D., and Anders, M.O. Eds., Pergamon Press, New York, 1991. With permission.)

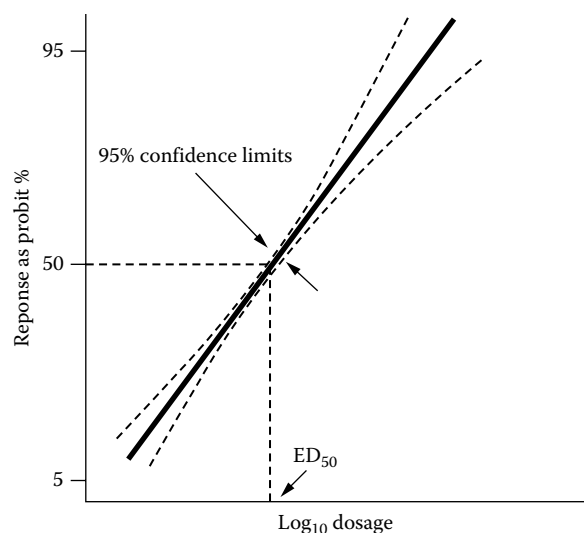


FIGURE 22.14 Dosage–mortality curve showing 95% confidence limits. These limits show the uncertainty associated with the dose–response curve resulting from the use of small numbers of animals. The confidence limits are narrowest at the ED_{50} (or LD_{50}). (From Ballantyne, B., *Exposure-dose-response relationships*, Chapter 3, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.)

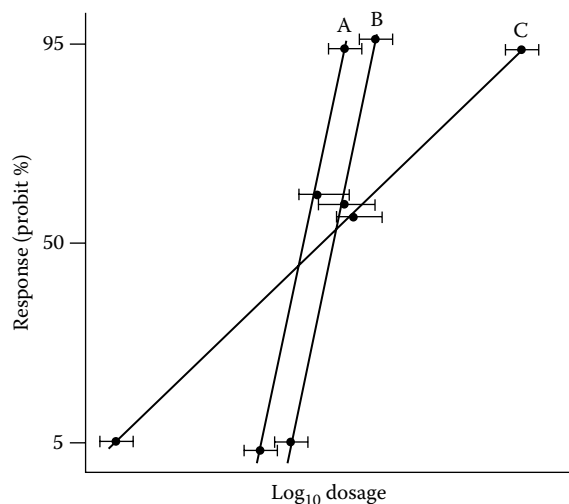


FIGURE 22.15 Influence of slopes of dosage–mortality data on the interpretation of LD_{50} data. All three materials (A, B, and C) have overlapping 95% confidence limits at the 50% response level and are therefore of comparable LD_{50} . Materials A and B have parallel dose–response lines and overlapping confidence limits at 5% and 95%; therefore, these two materials are of comparable lethal toxicity over a wide range of doses. Material C with a shallower slope has significantly different LD_5 and LD_{95} values and, therefore over a wide range of doses, has a differing lethal toxicity to materials A and B. With materials A and B, due to the steep slope of the dose–response line, a much larger proportion of the population will be affected by small incremental increases in dosage. With material C, there may be a greater hazard for the hyperreactive groups, because the LD_5 lies at a much lower dosage than for A and B. (From Ballantyne, B., *Exposure-dose-response relationships*, Chapter 3, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.)

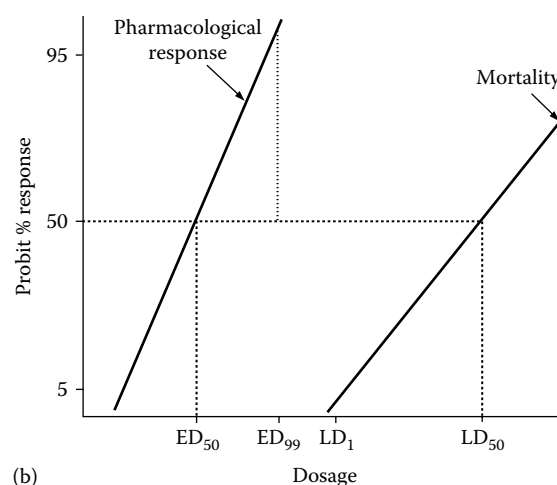
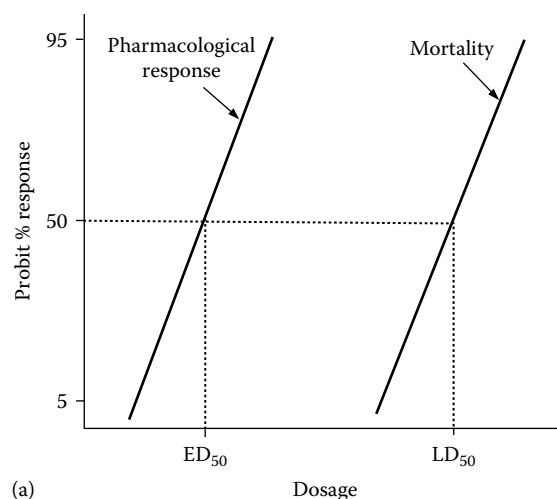


FIGURE 22.16 A simplistic method for assessing “safety ratios” for drugs is by comparing the ratio of the therapeutically ED_{50} and LD_{50} ; this ratio of LD_{50}/ED_{50} is referred to as the TI_{50} . For parallel pharmacological effect and lethality dose–response lines, the TI_{50} will be similar over a wide range of doses (a). The TI_{50} may be misleading if the dose–response lines for pharmacological and lethal effects are not parallel (b). As shown in this figure, the margin based on LD_{50} and ED_{50} may be reasonable. However, due to the shallow slope of the mortality dose–response line, the TI_{50} will be significantly lower at the 1% and 5% level; thus, the hyperreactive group may be at greater risk. In such a case, a better index of safety will be the ratio of the LD_1/ED_{99} , which is referred to as the margin of safety. (From Ballantyne, B., *Exposure-dose-response relationships*, Chapter 3, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.)

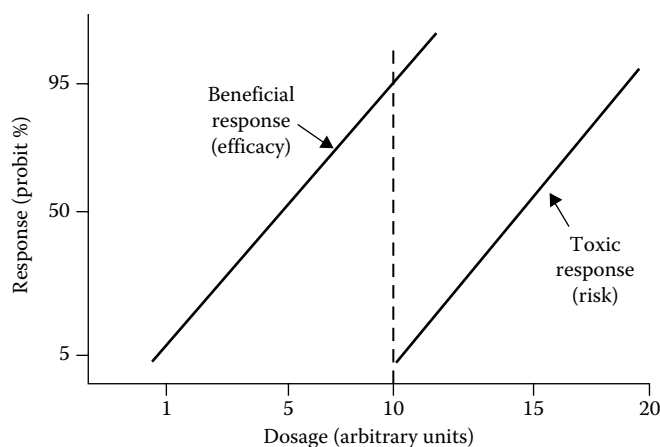


FIGURE 22.17 In situations where the beneficial (desired) dose–response curve of a pharmaceutical overlaps the toxic (side effect) dose–response curve of the drug, risk benefit determinations are made to assess the safety of the drug for its intended use. In making such decisions, many factors must be considered including the necessity for the drug, its intended use, and the type and severity of the side effect associated with the drug. For example, if the drug has some life-saving effect but produces nausea, the maximum dosage might be greater than 10 units to ensure efficacious treatment. Some patients would have to tolerate the nausea. An example of such a drug would be a chemotherapeutic agent. Conversely, nausea would not be tolerated if associated with a decongestant. For such a drug, the maximum dose would be kept below 10 units. (Adapted from Lowrance, W.M., *Of Acceptable Risk: Science and the Determination of Safety*, William Kaufmann, Los Altos, CA, 1976, Chapter 3.)

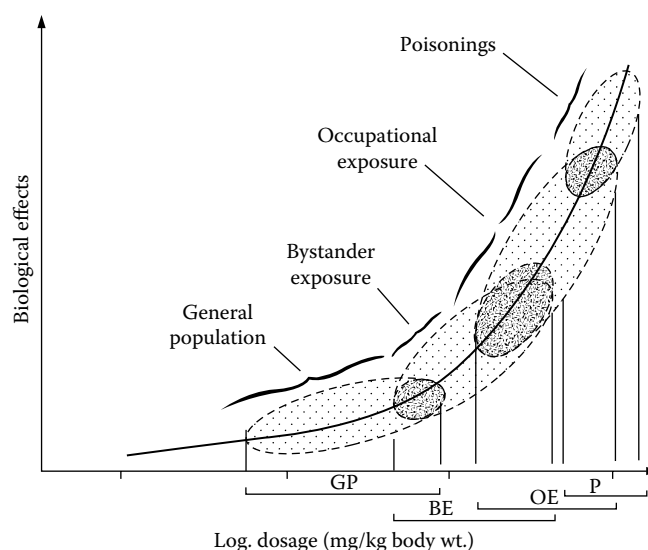


FIGURE 22.18 A theoretical dose–response relationship indicating the range of dosage (mg/kg of body weight) of a toxicant to which representative human populations might be exposed. Poisonings (P) tend to occur over a relatively narrow range, whereas in occupational exposures (OE) the range would be somewhat broader. Overlapping with OE, the dosage range to which bystanders would be exposed (BE) would be broader still, whereas the general population (GP) would encounter a dosage range of possibly one to three orders of magnitude. (From Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 2. With permission.)

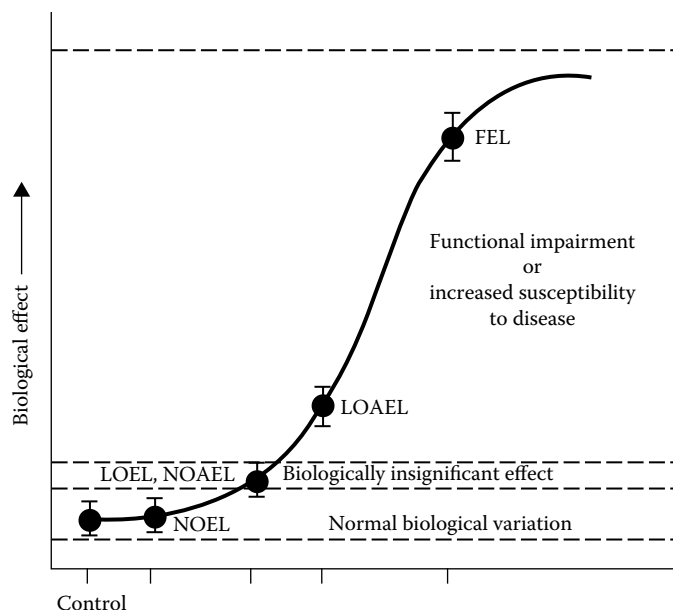


FIGURE 22.19 A dose–response curve from a typical toxicological study showing dose-related indices commonly used in risk assessment. A well-designed study should include dose levels that produce a frank effect level (FEL), a lowest observable adverse effect level (LOAEL), and either a lowest observable effect level (LOEL), a no observable adverse effect level (NOAEL), or a no observable effect level (NOEL). A FEL is a dose or exposure level that produces unmistakable *adverse* health effects that cause functional impairment or increased susceptibility to disease; a LOAEL is the lowest dose or exposure level that produces an *adverse* health effect; a LOEL is the lowest dose or exposure level that produces an observable effect, but not to a degree that would be expected to have a significant impact on the health of the animal (the LOEL is sometimes confused with a LOAEL); a NOAEL is the highest dose or exposure level at which no *adverse* health effects are observed, which are capable of functional impairment or increase susceptibility to disease (the NOAEL can be equivalent to the LOEL); a NOEL is the highest dose or exposure level at which no effects are observed outside of the range of normal biological variation for the species and strain under study. The effect, if any, observed at the NOEL should not be statistically significant when compared with the control group. (Adapted from Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 7.)

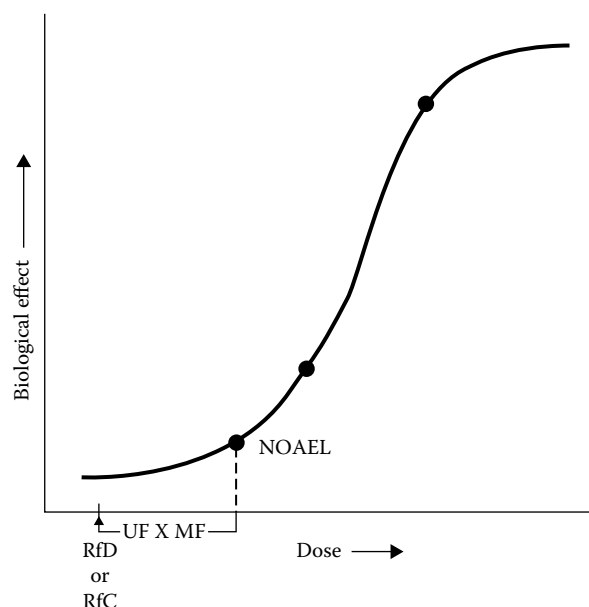


FIGURE 22.20 A dose–response curve from a typical toxicology study showing the relationship of the reference dose (RfD) and reference concentration (RfC) to the NOAEL. The RfD and RfC are determined from data developed from a subchronic or chronic animal study and represent an estimate of the level of exposure to which the human population (including sensitive subpopulations) can be exposed during a lifetime without deleterious effects. The RfD and RfC are used by EPA for noncarcinogenic effects having a threshold. The RfD and RfC are derived by dividing the NOAEL by the product of several uncertainty factors (UFs) and, prior to 2004, a modifying factor (MF) (see Table 22.53 for a description UF and MF). Depending on the degree of uncertainty associated with the data, the RfD and RfC can span several orders of magnitude from the NOAEL. The reference values (RfD and RfC) are similar in concept to FDA's acceptable daily intake (ADI) and OSHA's permissible exposure limit (PEL).

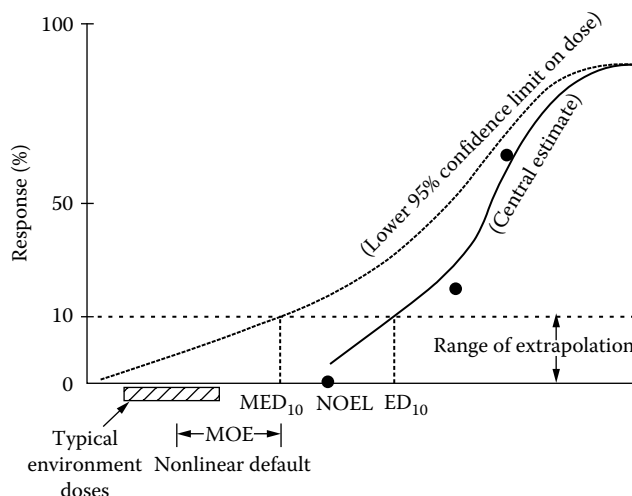


FIGURE 22.21 An alternative approach to the NOAEL method of deriving RfDs is the benchmark dose (BMD) method. In this approach, an effect level (usually between 1% and 10% response rate) is identified from the dose–response curve (solid line) and a bounding estimate on that dose derived (dotted line). A bound on the dose evoking the response of interest is identified and this value, the BMD is once again divided by appropriate UFs to derive an RfD or concentration. In this example, the dose causing a 10% response is chosen (ED_{10}), and a bounding estimate (dotted line) on this response is used to derive the BMD or minimum effective dose (MED_{10}). The MED_{10} is more often currently referred to as the LED_{10} for the lower limit on ED_{50} . The MED_{10}/LED_{10} is then divided by an UF to derive the RfD. In this case, the margin of exposure (MOE) is shown indicating where the MED_{10}/LED_{10} is found in relation to the typical environmental exposure. (From Gargas, M.L. et al., *Environmental health risk assessment: Theory and practice*, Chapter 82, in *General and Applied Toxicology*, Ballantyne, B., Marrs, T.C., and Syversen, T. Eds., Grove's Dictionary, New York, 1999. With permission.)

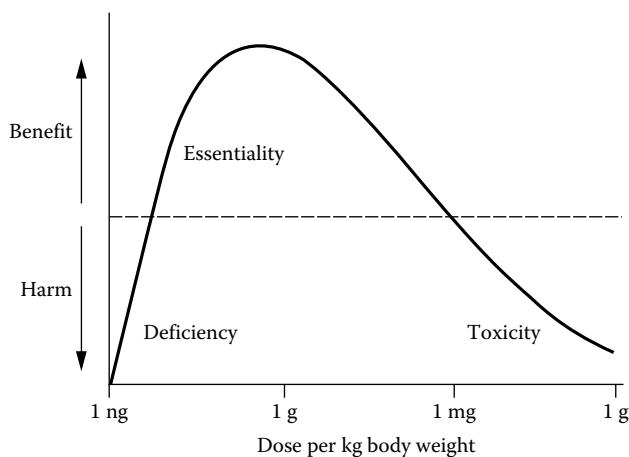


FIGURE 22.22 Harmful effects associated with a substance (deficiency and toxicity) can be produced at both low and high doses. Examples of such materials include metals that act as cofactors for essential biochemical processes. Note the wide range in the dosage scale. Because of the large UFs associated with risk assessment, the estimated safe exposure levels of such substances may fall at or near the levels that result in deficiency. This emphasizes the sometimes ultraconservative approaches used in the risk assessment process. (Modified from Crone, H.D., *Chemicals and Society: A Guide to the New Chemical Age*, Cambridge University Press, Cambridge, U.K., 1986, Chapter 4.)

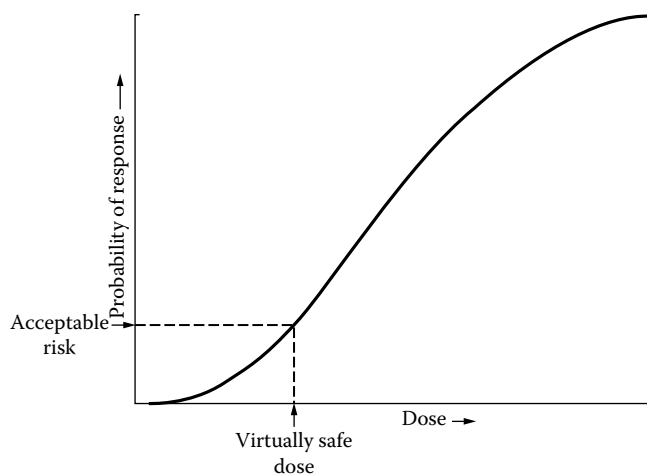


FIGURE 22.23 The determination of a virtually safe dose (VSD). Applied by regulatory agencies for carcinogens that are assumed not to have a threshold, the VSD is defined as the exposure level that is not expected to produce an excess of cancers beyond that which is deemed acceptable to society (acceptable risk). Acceptable risk is generally considered to be 1×10^{-6} (one in one million) but can range between 10^{-4} and 10^{-7} depending on the chemical of concern and the circumstances of the exposure. (Reprinted from *Food Cosmet. Toxicol.*, 19, Munro, I.C. and Krewski, D.R., Risk assessment and regulatory decision making, 549, Copyright 1981, with permission from Elsevier.)

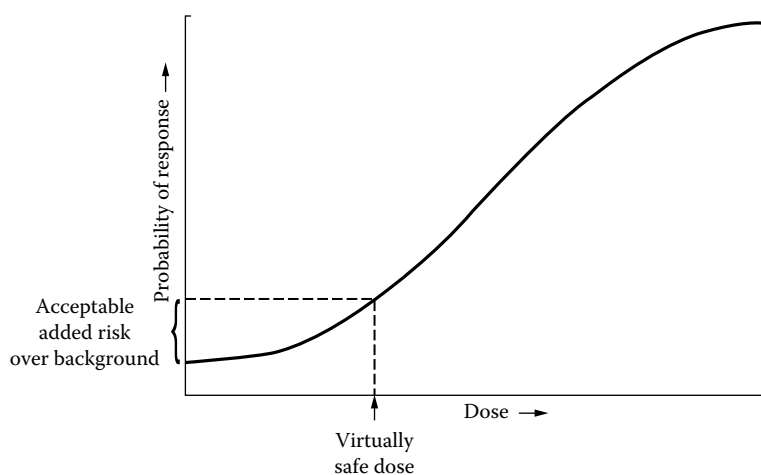


FIGURE 22.24 The determination of a VSD in the presence of background. (Reprinted from *Food Cosmet. Toxicol.*, 19, Munro, I.C. and Krewski, D.R., Risk assessment and regulatory decision making, 549, Copyright 1981, with permission from Elsevier.)

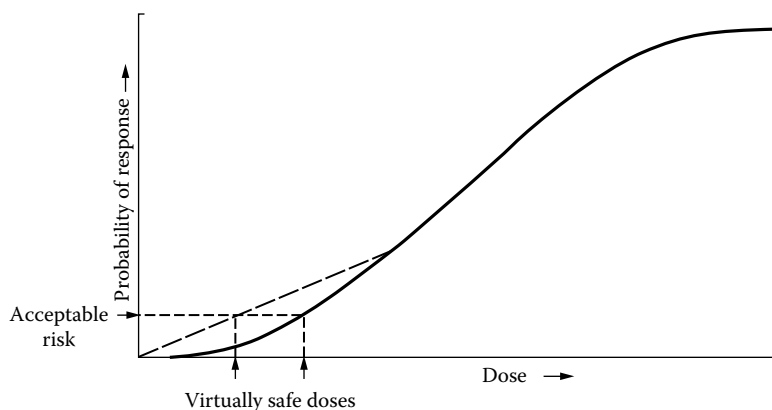


FIGURE 22.25 The effect of linear extrapolation of a sublinear dose–response curve on the determination of a VSD. (Reprinted from *Food Cosmet. Toxicol.*, 19, Munro, I.C. and Krewski, D.R., Risk assessment and regulatory decision making, 549, Copyright 1981, with permission from Elsevier.)

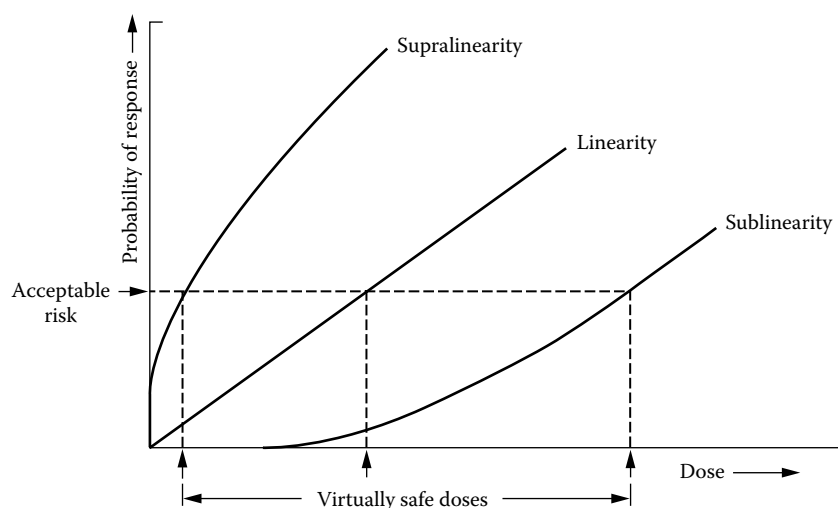


FIGURE 22.26 The effect of the shape of the dose-response curve on the determination of a VSD. (Reprinted from *Food Cosmet. Toxicol.*, 19, Munro, I.C. and Krewski, D.R., Risk assessment and regulatory decision making, 549, Copyright 1981, with permission from Elsevier.)

TABLE 22.8

Mathematical Models Used in the Determination of Low-Dose-Response Relationships for Potentially Carcinogenic Agents

Model	Description	Probability of a Test Animal Responding at Dose Level "d"	Shape of Parameter	Low-Dose Response
One hit	Based on the concept that a response will occur after the target has been "hit" by a single biologically effective unit of dose	$1 - \exp^{-\beta d}$	$\beta > 0$	Linear at low doses
Multihit	Based on an extension of the one-hit model assuming that more than one hit is required to induce a response	$\beta d \int_0^d (u^{k-1} e^{-u} / \Gamma(k)) du$	$k > 0$ $\beta > 0$	Linear at low doses only when the shape parameters are equal to unity
Weibull		$1 - \exp^{-\beta d^m}$	$m > 0$ $\beta > 0$	When shape parameters are curves approaching zero at slower than linear or sublinear rate
Multistage	Based on the assumption that the induction of irreversible self-replicating toxic effects is the result of a number of random biological events, the time of each being in strict linear proportion to the dose rate	$1 - \exp - \sum_{i=L}^k \alpha_i d^i$	k , an integer $\alpha_i > 0$ $i = 1 \rightarrow k$	Linear at low doses only when the linear coefficient B_1 is positive; the relationship is sublinear otherwise

Sources: Data from Van Ryzin, J., *J. Occup. Med.*, 22, 321, 1980; Van Ryzin, J. and Rai, K., The use of quantal response data to make predictions, in *The Scientific Basis of Toxicity Assessment*, Witschi, H.R. Ed., Elsevier/North-Holland, New York, p. 273, 1980; Munro, I.C. and Krewski, D.R., *Food Cosmet. Toxicol.*, 19, 549, 1981; Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 7. With permission.

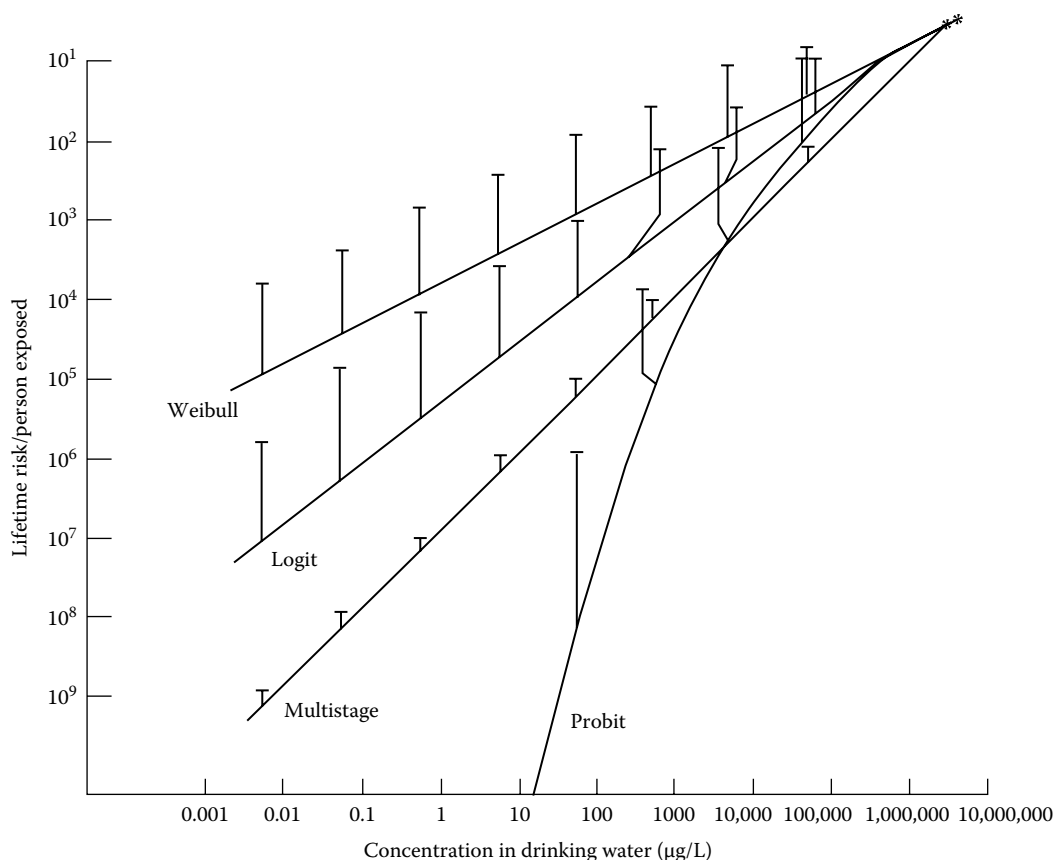


FIGURE 22.27 Dose–response curves extrapolated with data from a chronic bioassay (represented by the starred points in the upper right-hand corner of the figure) using the Weibull, Logit, Multistage, and Probit models. The curves are drawn from the point estimates with the vertical bars indicating the upper 95% confidence limit. The lower 95% confidence limit is the horizontal axis. This figure demonstrates the degree of variability (as much as several orders of magnitude) that can occur between the curves generated by the various models. (Modified from U.S. Environmental Protection Agency, *Techniques for the Assessment of the Carcinogenic Risk to the U.S. Population due to Exposure from Selected Volatile Organic Compounds from Drinking Water via the Ingestion, Inhalation and Dermal Routes*, Cothern, C.R., Coniglio, W.A., and Marcus, W.L. Eds., Office of Drinking Water, NTIS, PB 84-213941, 1984.)

EPIDEMIOLOGY

Epidemiology, the study of the distribution and determinants of disease in human populations, is one of the tools used in the hazard identification step of the risk assessment process (see Figure 22.1). Despite the many problems inherent to epidemiological studies including various biases, confounding factors, and inadequate quantitation of exposures, these studies offer a major advantage over those conducted with animals: the direct observation of effects in humans.

Much of the uncertainty associated with risk assessment results from the extrapolation of animal data to humans. Quality epidemiological studies can significantly reduce or eliminate such uncertainty. For this reason, human data are preferred over animal data for risk assessment (see Table 22.1). Usually, however, the availability of quality epidemiological studies is limited, and human, animal, and

in vitro data are used together in a “weight-of-evidence” approach to the risk assessment process.

Most toxicologists will encounter epidemiological data at some point in their careers. Epidemiology has its own unique methodology, measurement indices, and terminology. Much of the information from these studies is reported as relative risks, rates, ratios, and proportions. This section provides the toxicologist with an overview of epidemiological study designs, their uses and limitations, and the type of information they provide. In addition, general information on incidence and causation is provided for human cancer and reproductive and developmental effects, end points frequently the focus of epidemiological studies. Knowledge of such information is useful for evaluating the design and interpreting the results of human studies (Figures 22.28 and 22.29; Tables 22.9 through 22.28).

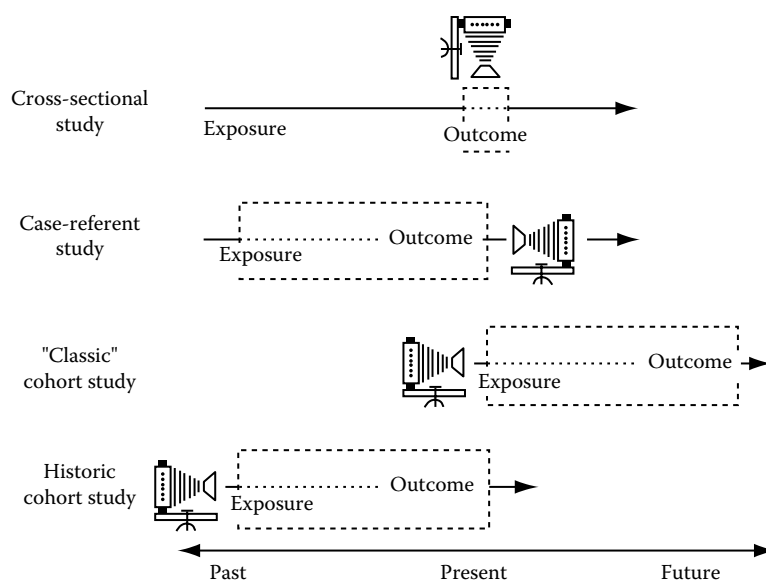


FIGURE 22.28 Common epidemiological study designs. The cross-sectional study takes a snapshot of both exposure and outcome at a particular point in time. In the case-referent study, the investigator identifies the outcome and looks back at the exposure. The cohort study can be done from two perspectives. Both approaches identify exposure groups and follow them to observe outcomes; however, one is done historically on past events, and the other identifies current exposures and follows the study members forward. (From Selevan, S.G., Epidemiology, Chapter 9, in *Occupational and Environmental Reproductive Hazards: A Guide for Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993. With permission.)

TABLE 22.9
Human Data Commonly Used in Risk Assessment

Study Type	Alternative Terms	Comments on Use
Cross-sectional	Prevalence, survey	Sampling of a population at a given point in time to assess prevalence of a disease. Most useful for studying chronic diseases of high frequency. Cannot measure incidence. Although associations may be drawn with prevalent cases, the temporal and causal order of such associations cannot be determined
Case-control	Retrospective, dose or case-referent, case history	Compares previous exposure in subjects with disease with one or more groups of subjects without disease. Selection of cases and noncases can be controlled. Exposures cannot be controlled. If exposure data available, a NOEL may be identified. Exposure history may be difficult to reconstruct outside of an occupational setting. Recall and other biases are possible due to retrospective evaluation. Allows estimation of relative odds of exposure in cases and controls but not absolute risk
Cohort	Longitudinal, prospective, incidence	Population or sample of subjects at risk of disease observed through time for outcome of interest. May fail to detect rare outcome. Many factors can be controlled for reduced bias (prospective design). Dose-response curves may be constructed if dose or exposure data available. Allows estimation of absolute and relative risk
Clinical trials		Type of cohort study in which investigator controls treatment (exposure). Generally not applicable to environmental issues. Intervention trials in which an exposure is removed or changed (e.g., medication, smoking, diet) are useful for evaluating causality
Experimental studies		Controlled human exposures generally of low dose and limited exposure time. Used for hazard identification, dose-response, and risk characterization
Case reports		Suggests nature of acute end points. Cannot be used to support absence of hazard

Sources: Modified from Piantadose, S., Epidemiology and principles of surveillance regarding toxic hazards in the environment, Chapter 6, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992; U. S. Environmental Protection Agency, *Interim Methods for the Development of Inhalation Reference Doses*, Blackburn, K., Dourson, M., Erdreich, L., Jarabek, A.M., and Overton, J. Jr. Eds., Environmental Criteria and Assessment Offices, EPA1600/8-88/066F, 1989.

TABLE 22.10
Epidemiological Terms

Annual crude death rate	$= \frac{\text{Total number of deaths during a given year}}{\text{Total population at midyear}} \times 1000$
Annual specific death rate	$= \frac{\text{Total number of deaths in a specific group during a given year}}{\text{Total population in the specific group at mid year}} \times 100$
Proportional mortality rate	$= \frac{\text{Total number of deaths in a specific group}}{\text{Total number of deaths}} \times 100$
Infant mortality rate (IMR)	$= \frac{\text{Infant deaths}}{\text{Total live births}} \times 1000$
Standard mortality ratio (SMR)	$= \frac{\text{Observed deaths}}{\text{Expected deaths}}$
Cause-of-death ratio	$= \frac{\text{Deaths from a specific cause over a period of time}}{\text{Total deaths due to all causes in the same time period}} \times 100$
Incidence rate	$= \frac{\text{Number of new cases over a period of time}}{\text{Population at risk over the same time period}}$
Prevalence rate	$= \frac{\text{Number of existing cases at a point in time}}{\text{total population}}$
Relative risk (risk ratio)	$= \frac{\text{Incidence among the exposed}}{\text{Incidence among the nonexposed}}$
Attributable risk (risk difference)	$= \text{Incidence among the exposed} - \text{Incidence among the nonexposed}$
Relative odds ratio	$= \frac{\text{Number of exposed individuals with disease}}{\text{Number of exposed individuals without disease}} \times \frac{\text{Number of nonexposed individuals without disease}}{\text{Number of nonexposed individuals with disease}}$

Sources: Selevan, S.G., Epidemiology, Chapter 9, in *Occupational and Environmental Reproductive Hazards: A Guide for Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993; Hallenbeck, W.H. and Cunningham, K.M., Qualitative evaluation of human and animal studies, Chapter 3, in *Quantitative Risk Assessment for Environmental and Occupational Health*, Lewis Publishers, Chelsea, MI, 1986; Gamble, J.F. and Battigelli, M.C., Epidemiology, Chapter 5, in *Patty's Industrial Hygiene and Toxicology*, 3rd revised edn., Vol. I., Clayton, G.D. and Clayton, F.E. Eds., John Wiley & Sons, New York, 1978.

TABLE 22.11

Steps in the Investigation of a Cancer Cluster

1. Each case of cancer must be identified and confirmed. Histopathology and medical records should be reviewed for accuracy of diagnosis
2. The time of occurrence of the cancer case should be identified. Employees working for the company during this time should be evaluated. This step of the investigation can be affected by a changing workforce
3. The observed number of cases should be compared with the expected number of cases. The expected number of cases may be obtained from cancer registries. This comparison should be age standardized, because the majority of cancer occurs in older individuals. Comparison of the observed number of cases with the expected number allows for the calculation of a standard mortality ratio (SMR). An SMR greater than one may indicate an excess of cancer
4. After the types of cancer are identified, determination should be made of whether a particular cell type or target organ is overrepresented in the population being studied
5. The latency period must be evaluated. Latency is the time from the onset of exposure to a certain chemical or environment to appearance of clinical disease. The latency period for solid tumors is 15–20 years, and for hematological tumors, 5–10 years. If an individual is noted to have cancer after a recent exposure, then the latency period is too short to implicate the chemical or the environment as the cause
6. Confounding and contributing factors must be evaluated. These include smoking, substance and drug abuse, a family history of cancer, multiple exposures to other chemicals or potential carcinogens, or employment in an industry that has been associated with carcinogenesis
7. There may be a particular job or site in an area of industry that is overrepresented in cases of cancer. The SMR may not show an elevation of cancer cases in the industry overall, but specific jobs or sites may show an excess of deaths if separate SMRs are calculated for these sites and job descriptions
8. If an excess of similar cases exists, then the issue of causation must be addressed. A chemical may be responsible, and further investigation is warranted. If there is a mixture of cancer types, then this is unlikely to be a true cancer cluster. If there is overrepresentation by a particular job, environment, or chemical exposure, then a cluster may indeed be occurring. If there is no pattern of common exposures or job site locations, then the chances of a cluster are diminished
9. An environmental assessment and industrial hygiene review is warranted to determine whether exposures to potential carcinogens are occurring. Previous environment surveys should be reviewed and appropriate environmental monitoring conducted as needed
10. The investigation should conclude that
 - a. Cluster is not present
 - b. A cluster may be present but is inconsistent with an occupational or environmental exposure cause
 - c. A cluster is present and could be related to a chemical or environmental exposure
 - d. The cluster is definitely related to the exposure
 If an exposure or occupational setting is implicated, then aggressive corrective action must be undertaken

Source: From Piantadosi, S. and Sullivan, J.B., Chemical and environmental carcinogenesis, Chapter 8, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.

TABLE 22.12

Overall Evaluation of Human and Animal Evidence about the Carcinogenicity of 597 Agents^a

Animals	Humans				Total
	Sufficient	Limited	Inadequate	No Adequate Data	
Sufficient	21 (3.5)	12 (2.0)	47 (7.9)	123 (20.6)	203 (34.0)
Limited	8 (1.3)	1 (0.2)	28 (4.7)	124 (20.8)	161 (27.0)
Inadequate	6 (1.0)	1 (0.2)	33 (5.5)	164 (27.5)	204 (34.2)
No adequate data	1 (0.2)	0	1 (0.2)	24 (4.0)	26 (4.4)
Lack of carcinogenicity	0	0	1 (0.2)	2 (0.3)	3 (0.5)
Total	36 (6.0)	14 (2.3)	110 (18.4)	437 (73.2)	597 (100.0)

Sources: Data from IARC Working Group on the Evaluation of Carcinogenic Risk, Overall evaluations of carcinogenicity: An updating of IARC monographs, in *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 1–42(Suppl 7), IARC Working Group on the Evaluation of Carcinogenic Risk, Lyon, France, 1987; Meijers, J.M.M. et al., *Regul. Toxicol. Pharmacol.*, 16, 215, 1992. With permission.

^a Classified are 597 agents from the 1987 IARC database. As can be seen for 36 agents (6%), sufficient evidence of carcinogenicity in humans exists. Of the 36 human carcinogens, 15 (41.7%) did not show sufficient evidence of carcinogenicity in animals. For one of these agents, no adequate animal data existed. In contrast, for 203 agents (34%), sufficient evidence of carcinogenicity in experimental animals is available. Only 21 of these 203 agents (10.3%) also proved to be a human carcinogen. This is partly due to the fact that for 123 agents, no adequate human data existed. Nevertheless, for 59 of the 203 agents (29%), the existing human evidence resulted in an evaluation that differed from the categorization based on animal evidence. The majority of agents that seemed to be carcinogenic in animals did not seem to produce clear and positive results in humans. For agents with sufficient evidence based on human epidemiology, the correspondence with animal experimental data is weak.

TABLE 22.13
Estimates on Causes of Cancer

Factor	USA, 1981		Birmingham England, 1979 Estimate (%)		USA, 1977 Estimate (%)	
	Estimate (%)	Range (%)	Male	Female	Male	Female
Tobacco	30	25–40	30	7	28	8
Alcohol	3	2–4				
Tobacco/alcohol			5	3	4	1
Diet	35	10–70			40	57
Life style			30	63		
Food additives	<1	–5 to 2				
Reproduction and sexual behavior	7	1–13				
Occupation	4	2–8	6	2	4	2
Industrial products	<1	<1–2				
Pollution	2	<1–5				
Sunlight			10	10		
Ionizing radiation					8	8
Geophysical factors	3	2–4	1	1		
Iatrogenic (drugs and medical procedures)	1	0.5–3	1	1		
Infection	10?	1–?				
Exogenous hormones					—	4
Congenital			2	2		
					16	20
Unknown			15	11		

Sources: Data from Doll, B. and Peto, R., *J. Natl. Cancer Inst.*, 68, 1191, 1981; Higginson, J. and Muir, C.S., *J. Natl. Cancer Inst.*, 63, 1291, 1979; Wynder, E.L. and Gori, G.B., *J. Natl. Cancer Inst.*, 58, 825, 1977; Weisburger, E.K., Industrial and environmental cancer risks, in *Dangerous Properties of Industrial Materials*, Section 3, 6th edn., Sax, N.I. Ed., Van Nostrand Reinhold, New York, 1984. With permission.

Note: ?, values uncertain.

TABLE 22.14
Top 12 Most Frequently Observed Site-Specific Cancers in Humans in the United States for the 2-Year Period 1986–1987 with Top 10 Estimated Ranking for Year 2012

Rank	Males and Females		Males		Females	
	Site	Rate ^a	Site	Rate ^a	Site	Rate ^a
1	Breast	61.3	(1) ^b Prostate gland	94.2	(1) ^b Breast	112.1
2	Lung and bronchus	56.6	(2) Lung and bronchus	82.0	(3) Colon/rectum	41.7
3	Colon/rectum	49.8	(3) Colon/rectum	61.2	(2) Lung and bronchus	38.1
4	Prostate gland	38.2	(4) Urinary bladder	32.4	(4) Cervix uteri, corpus, and uterus	30.0
5	Urinary bladder	18.1	(7) Non-Hodgkin's lymphoma	17.1	(9) Ovary	13.9
6	Cervix uteri, corpus, and uterus	16.3	(8) Oral and pharynx	16.4	(7) Non-Hodgkin's lymphoma	11.0
7	Non-Hodgkin's lymphoma	13.8	(9) Leukemia	13.0	(6) Melanoma of skin	10.0
8	Melanoma of skin	11.2	(5) Melanoma of skin	12.9	(10) Pancreas	7.6
9	Oral and pharynx	10.9	(6) Kidney	11.7	Urinary bladder	7.6
10	Leukemia	9.7	Stomach	10.6	Leukemia	7.4
11	Pancreas	8.8	(10) Pancreas	10.6	(5) Thyroid gland	6.1
12	Kidney	8.4	Larynx	8.2	Brain and nervous system	5.8
					(8) Kidney	

Sources: Data from Ries, L.A.G. et al., *Cancer Statistics Review, 1973–1987*, National Cancer Institute, Bethesda, MD, 1990; American Cancer Society, *Cancer Facts and Figures*, American Cancer Society, Okaland, CA, 2012; Adapted from Huff, J. et al., *Environ. Health Perspect.*, 93, 247, 1991.

^a Age adjusted rates per 100,000 population 1986–1987.

^b Values in brackets are relative ranking for males and females based on year 2012 estimates. Kidney not ranked in top 12 for women in 1986–1987.

TABLE 22.15

Probability (%) of Developing Invasive Cancers over Selected Age Intervals, by Sex, United States, 1994–1996 versus 2006–2008^a

		Birth to 39	40–59	60–79	60–69	70 and Older	Birth to Death
All sites ^b	Male	1.61 (1 in 62)	8.17 (1 in 12)	33.65 (1 in 3)	—	—	43.56 (1 in 2)
		1.45 (1 in 69)	8.68 (1 in 12)	— ^c	16.00 (1 in 6)	38.27 (1 in 3)	44.85 (1 in 2)
	Female	1.94 (1 in 52)	9.23 (1 in 11)	22.27 (1 in 4)	—	—	38.11 (1 in 3)
		2.15 (1 in 46)	9.10 (1 in 11)	—	10.34 (1 in 10)	26.68 (1 in 4)	38.08 (1 in 3)
Bladder (urinary) ^c	Male	—	—	—	—	—	—
		0.02 (1 in 5,035)	0.38 (1 in 266)	—	0.92 (1 in 109)	3.71 (1 in 27)	3.84 (1 in 26)
	Female	—	—	—	—	—	—
		0.01 (1 in 12,682)	0.12 (1 in 851)	—	0.25 (1 in 400)	0.98 (1 in 102)	1.15 (1 in 87)
Breast	Female	0.43 (1 in 235)	4.06 (1 in 25)	6.88 (1 in 15)	—	—	12.56 (1 in 8)
		0.49 (1 in 203)	3.76 (1 in 27)	—	3.53 (1 in 28)	6.58 (1 in 15)	12.29 (1 in 8)
Colon and rectum	Male	0.06 (1 in 1,579)	0.85 (1 in 124)	3.97 (1 in 29)	—	—	5.64 (1 in 18)
		0.08 (1 in 1,236)	0.92 (1 in 109)	—	1.44 (1 in 70)	4.32 (1 in 23)	5.27 (1 in 19)
	Female	0.05 (1 in 1,947)	0.67 (1 in 149)	3.06 (1 in 33)	—	—	5.55 (1 in 18)
		0.08 (1 in 1,258)	0.73 (1 in 137)	—	1.01 (1 in 99)	3.95 (1 in 25)	4.91 (1 in 20)
Leukemia	Male	—	—	—	—	—	—
		0.16 (1 in 614)	0.22 (1 in 445)	—	0.34 (1 in 291)	1.24 (1 in 81)	1.57 (1 in 64)
	Female	—	—	—	—	—	—
		0.14 (1 in 737)	0.15 (1 in 665)	—	0.21 (1 in 482)	0.81 (1 in 123)	1.14 (1 in 88)
Lung and bronchus	Male	0.04 (1 in 2,592)	1.29 (1 in 78)	6.35 (1 in 16)	—	—	8.11 (1 in 12)
		0.03 (1 in 3,631)	0.91 (1 in 109)	—	2.26 (1 in 44)	6.69 (1 in 15)	7.66 (1 in 13)
	Female	0.03 (1 in 2,894)	0.94 (1 in 106)	3.98 (1 in 25)	—	—	5.69 (1 in 18)
		0.03 (1 in 3,285)	0.76 (1 in 132)	—	1.72 (1 in 58)	4.91 (1 in 20)	6.33 (1 in 16)
Melanoma of the skin ^d	Male	—	—	—	—	—	—
		0.15 (1 in 677)	0.63 (1 in 158)	—	0.75 (1 in 133)	1.94 (1 in 52)	2.80 (1 in 36)
	Female	—	—	—	—	—	—
		0.27 (1 in 377)	0.56 (1 in 180)	—	0.39 (1 in 256)	0.82 (1 in 123)	1.83 (1 in 55)
Non-Hodgkin's lymphoma	Male	—	—	—	—	—	—
		0.13 (1 in 775)	0.45 (1 in 223)	—	0.60 (1 in 167)	1.77 (1 in 57)	2.34 (1 in 43)
	Female	—	—	—	—	—	—
		0.09 (1 in 1,152)	0.32 (1 in 313)	—	0.44 (1 in 228)	1.41 (1 in 71)	1.94 (1 in 51)
Prostate	Male	<1 in 10,000	1.90 (1 in 53)	13.69 (1 in 7)	—	—	15.91 (1 in 6)
		0.01 (1 in 8,499)	2.63 (1 in 38)	—	6.84 (1 in 15)	12.54 (1 in 8)	16.48 (1 in 6)
Uterine cervix	Female	—	—	—	—	—	—
		0.15 (1 in 650)	0.27 (1 in 373)	—	0.13 (1 in 771)	0.18 (1 in 549)	0.68 (1 in 147)
Uterine corpus	Female	—	—	—	—	—	—
		0.07 (1 in 1,373)	0.77 (1 in 130)	—	0.87 (1 in 114)	1.24 (1 in 81)	2.61 (1 in 38)

Sources: Adapted from American Cancer Society, *Cancer Facts and Figures*, American Cancer Society, Okaland, CA, 2000; American Cancer Society, *Cancer Facts and Figures*, American Cancer Society, Okaland, CA, 2012.

1994–1996 Data source: DEVCAN Software, Version 4.0, Surveillance, Epidemiology and End Results Program, 1973–1996; Division of Cancer Control and Population Sciences, National Cancer Institute. 2006–2008; Probability of Developing or dying of Cancer Software, Version 6.6.0 Statistical Research and Applications Branch, National Cancer Institute, 2011.

^a Of those free of cancer at beginning of age interval. Based on cancer cases diagnosed during 1994–1996 or 2006–2008. The “1 in” statistic and the inverse of the percentage may not be equivalent due to rounding. **Bold, 2006–2008**; non-bold, 1994–1996.

^b Excludes basal and squamous cell skin cancers and *in situ* carcinomas except urinary bladder.

^c Includes invasive and *in situ* cancer cases.

^d Statistic is for whites only.

^e —, Data not available.

TABLE 22.16**The First and Second Most Common Types of New Cancer Cases by World Area—Males 2008**

	Kaposi's Sarcoma	Liver	Lung and Bronchus	Prostate	Oral Cavity	Esophagus	Bladder	Stomach	Colon and Rectum
Africa									
Eastern	First					Second			
Middle		First		Second					
Northern			First				Second		
Southern			Second	First					
Western		First		Second					
Caribbean			Second	First					
Central America				First				Second	
South America			Second	First					
North America			Second	First					
Asia									
Eastern			First					Second	
South Eastern		Second	First						
South Central			First		Second				
Western			First						Second
Europe									
Central and Eastern			First						Second
Northern			Second	First					
Southern			Second	First					
Western			Second	First					
Australia/New Zealand				First					Second
Melanesia		Second			First				
Micronesia			First	Second					
Polynesia ^a			First	First					Second

Source: Adapted from American Cancer Society, *Global Cancer Facts and Figures*, 2nd edn., American Cancer Society, Atlanta, GA, 2011.

Data source: GLOBOCAN 2008. International Agency for Research on Cancer (<http://globocan.iarc.fr>).

^a Lung and prostate at equivalent incidence.

TABLE 22.17**The First and Second Most Common Types of New Cancer Cases by World Area—Females 2008**

	Lung and Bronchus	Colon and Rectum	Cervix Uteri	Breast	Thyroid
Africa					
Eastern			First	Second	
Middle			Second	First	
Northern			Second	First	
Southern			Second	First	
Western			Second	First	
Caribbean			Second	First	
Central America			Second	First	
South America			Second	First	
North America	Second			First	
Asia					
Eastern	Second			First	
South Eastern			Second	First	
South Central			First	Second	
Western		Second		First	
Europe					
Central and Eastern		Second		First	
Northern		Second		First	
Southern		Second		First	
Western		Second		First	
Australia/New Zealand		Second		First	
Melanesia			First	Second	
Micronesia		Second		First	
Polynesia				First	Second

Source: Adapted from American Cancer Society, *Global Cancer Facts and Figures*, 2nd edn., American Cancer Society, Atlanta, GA, 2011.

Data source: GLOBOCAN 2008 International Agency for Research on Cancer (<http://globocan.iarc.fr>).

TABLE 22.18

Known^a and Suspected^b Human Carcinogens
Agents, Substances, Mixtures, or Exposure Circumstances Known to Be Human Carcinogens

Aflatoxins
 Alcoholic beverage consumption
 4-Aminobiphenyl (4-aminodiphenyl)
 Analgesic mixtures containing phenacetin
 Aristolochic acids
 Arsenic and inorganic arsenic compounds
 Asbestos
 Azathioprine
 Benzene
 Benzidine
 Beryllium and beryllium compounds
 Bis(chloromethyl) ether and technical-grade chloromethyl methyl ether
 1,3-Butadiene
 Cadmium and cadmium compounds
 Chlorambucil
 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU)
 Chromium hexavalent compounds
 Coal tar and coal tar pitches
 Coke oven emissions
 Cyclophosphamide
 Cyclosporin A
 Diethylstilbestrol (DES)
 Dyes that metabolize to benzidine
 Erionite
 Estrogens, steroidal
 Ethylene oxide
 Formaldehyde
 Hepatitis B virus
 Hepatitis C virus
 Human papilloma viruses: some genital–mucosal types
 Melphalan
 Methoxsalen with ultraviolet A therapy
 Mineral oils: untreated and mildly treated
 Mustard gas
 2-Naphthylamine
 Neutrons
 Nickel compounds
 Radon
 Silica, crystalline (respirable size)
 Solar radiation and exposure to sunlamps or sunbeds
 Soots
 Strong inorganic acid mists containing sulfuric acid
 Tamoxifen
 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
 Thiotepa
 Thorium dioxide
 Tobacco smoke, tobacco smoking, tobacco-smokeless
 Ultraviolet radiation, broad spectrum
 Vinyl chloride
 Wood dust
 X-radiation and gamma radiation

TABLE 22.18 (continued)

Known^a and Suspected^b Human Carcinogens
Agents, Substances, Mixtures, or Exposure Circumstances Reasonably Anticipated to Be Human Carcinogens

Acetaldehyde
 2-Acetylaminofluorene
 Acrylamide
 Acrylonitrile
 Adriamycin
 2-Aminoanthraquinone
o-Aminoazotoluene
 1-Amino-2-methylantraquinone
 2-Amino-3,4-dimethylimidaz [4,5-*f*]quinoline
 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
 1-Amino-2-methylantraquinone
 2-Amino-3-methylimidazo[4,5-*f*]quinoline
 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
 Amitrole
o-Anisidine and its hydrochloride
 Azacitidine
 Basic red 9 monohydrochloride
 Benz[*a*]anthracene
 Benzo[*b*]fluoranthene
 Benzo[*j*]fluoranthene
 Benzo[*k*]fluoranthene
 Benzo[*a*]pyrene
 Benzotrifluoride
 2,2,-Bis(bromomethyl)-1,3-propanediol
 Bis(chloroethyl) nitrosourea
 Bromodichloromethane
 1,4-Butanediol dimethanesulfonate
 Butylated hydroxyanisole
 Captafol
 Carbon tetrachloride
 Ceramic fibers (respirable size)
 Chloramphenicol
 Chlorendic acid
 Chlorinated paraffins (C₁₂–60% chlorine)
 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
 Chloroform
 3-Chloro-2-methylpropene
 4-Chloro-*o*-phenylenediamine
 Chloroprene
p-Chloro-*o*-toluidine and *p*-chloro-*o*-toluidine hydrochloride
 Chlorozotocin
 Cisplatin
 Cobalt sulfate
 Cobalt-tungsten carbide: powders and hard metals
p-Cresidine
 Cupferron
 Dacarbazine
 Danthron
 DDT (dichlorodiphenyltrichloroethane)
 2,4-Diaminoanisole sulfate
 2,4-Diaminotoluene
 Diazoaminobenzene
 Dibenz[*a,h*]acridine
 Dibenz[*a,j*]acridine

TABLE 22.18 (continued)
Known^a and Suspected^b Human Carcinogens

Dibenz[*a,h*]anthracene
 7-H-Dibenzo[*c,g*]carbazole
 Dibenzo[*a,e*]pyrene
 Dibenzo[*a,h*]pyrene
 Dibenzo[*a,i*]pyrene
 Dibenzo[*a,l*]pyrene
 1,2-Dibromo-3-chloropropane
 1,2-Dibromoethane (ethylene dibromide)
 2,3-Dibromo-1-propanol
 1,4-Dichlorobenzene
 3,3-Dichlorobenzidine and 3,3-dichlorobenzidine dihydrochloride
 1,2-Dichloroethane
 Dichloromethane
 1,3-Dichloropropene (technical grade)
 Diepoxybutane
 Diesel exhaust particulates
 di(2-ethylhexyl) phthalate
 Diethyl sulfate
 Diglycidyl resorcinol ether
 3,3-Dimethoxybenzidine and 3,3-dimethoxybenzidine dihydrochloride
 4-Dimethylaminoazobenzene
 3,3'-Dimethylbenzidine
 Dimethylcarbamoyl chloride
 1,1-Dimethylhydrazine
 Dimethyl sulfate
 Dimethylvinyl chloride
 1,6-Dinitropyrene
 1,8-Dinitropyrene
 1,4-Dioxane
 Disperse blue 1
 Dyes metabolized to 3,3-dimethoxybenzidine
 (3,3-dimethoxybenzidine dye class)
 Dyes metabolized to 3,3-dimethylbenzidine (3,3-dimethylbenzidine
 dye class)
 Epichlorohydrin
 Ethylene thiourea
 Ethyl methanesulfonate
 Furan
 Glass wool fibers (inhalable), certain
 Glycidol
 Hexachlorobenzene
 Hexachloroethane
 Hexamethylphosphoramide
 Hydrazine and hydrazine sulfate
 Hydrazobenzene
 Indeno[1,2,3-*c,d*]pyrene
 Iron dextran complex
 Isoprene
 Kepone
 Lead and lead compounds
 Lindane, hexachlorohexane (technical grade) and other
 hexachlorocyclohexane isomers
 2-Methylaziridine (propyleneimine)
 5-Methylchrysene
 4,4-Methylenebis(2-chloroaniline) (MBOCA)

TABLE 22.18 (continued)
Known^a and Suspected^b Human Carcinogens

4,4-Methylenebis(*N,N*-dimethylbenzenamine)
 4,4'-Methylenedianiline and its dihydrochloride
 Methyleugenol
 Methyl methanesulfonate
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine
 Metronidazole
 Michler's ketone
 Mirex
 Naphthalene
 Nickel, metallic
 Nitrilotriacetic acid
o-Nitroanisole
 Nitrobenzene
 6-Nitrochrysene
 Nitrofen
 Nitrogen mustard hydrochloride
 Nitromethane
 2-Nitropropane
 1-Nitropyrene
 4-Nitropyrene
N-Nitrosodi-*n*-butylamine
N-Nitrosodiethanolamine
N-Nitrosodiethylamine
N-Nitrosodimethylamine
N-Nitrosodi-*n*-propylamine
N-Nitroso-*N*-ethylurea
 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)
N-Nitroso-*N*-methylurea
N-Nitrosomethylvinylamine
N-Nitrosomorpholine
N-Nitrosornicotine
N-Nitrosopiperidine
N-Nitrosopyrrolidine
N-Nitrososarcosine
o-Nitrotoluene
 Norethisterone
 Ochratoxin A
 4,4-Oxydianiline
 Oxymetholone
 Phenacetin
 Phenazopyridine hydrochloride
 Phenolphthalein
 Phenoxybenzamine hydrochloride
 Phenytoin and phenytoin sodium
 Polybrominated biphenyls
 Polychlorinated biphenyls
 Procarbazine and its hydrochloride
 Progesterone
 1,3-Propane sultone
 β-Propiolactone
 Propylene oxide
 Propylthiouracil
 Reserpine
 Riddelliine
 Safrole

(continued)

TABLE 22.18 (continued)**Known^a and Suspected^b Human Carcinogens**

Selenium sulfide
 Streptozotocin
 Styrene
 Styrene-7,8-oxide
 Sulfallate
 Tetrachloroethylene
 Tetrafluoroethylene
 Tetranitromethane
 Thioacetamide
 4,4'-Thiodianiline
 Thiourea
 Toluene diisocyanate
o-Toluidine and *o*-toluidine hydrochloride
 Toxaphene
 Trichloroethylene
 2,4,6-Trichlorophenol
 1,2,3-Trichloropropane
 Tris(2,3-dibromopropyl) phosphate
 Ultraviolet radiation A
 Ultraviolet radiation B
 Ultraviolet radiation C
 Urethane
 Vinyl bromide
 4-Vinyl-1-cyclohexene diepoxide
 Vinyl fluoride

Source: From U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program, *Annual Report on Carcinogens*, 12th Edition, 2011.

^a Known carcinogens are defined as those substances for which evidence from human studies indicates that there is a causal relationship between exposure to the substance and human cancer.

^b Suspected carcinogens (substances that may reasonably be anticipated to be human carcinogens) are defined as those substances for which there is limited evidence of carcinogenicity in humans or sufficient evidence of carcinogenicity in experimental animals.

TABLE 22.19**Reproductive End Points to Indicate Reproductive Dysfunction**

Sexual dysfunction: decreased libido; impotence
 Sperm abnormalities: decreased number; decreased motility; abnormal morphology
 Subfecundity: abnormal gonads; ducts of external genitalia; abnormal pubertal development; infertility of male or female origin; amenorrhea; anovulatory cycles; delay in conception
 Illness during pregnancy and parturition; toxemia; hemorrhage
 Early fetal loss (to 28 weeks)
 Late fetal loss (after 28 weeks) and stillbirth
 Intrapartum death
 Death in first week
 Decreased birth weight
 Change in gestational age at delivery: prematurity; postmaturity
 Altered sex ratio
 Multiple births
 Birth defects, major and minor
 Chromosome abnormalities, in fetal deaths, at amniocentesis, in perinatal deaths, in live births
 Infant death
 Childhood morbidity
 Childhood malignancies
 Age at menopause

} Perinatal death

Source: Warburton, I., Measurement of reproductive effects in human populations: Selected outcomes for study, in *Symposium on Criteria for Assessment of Health Effects at Chemical Disposal Sites*, Rockefeller University, New York, 1981. With permission.

TABLE 22.20**Possible Environmental Risk Factors for Spontaneous Abortion**

Factor(s)	Comment
Parental age	Advanced maternal age associated with increase in trisomic and chromosomally normal abortions
Socioeconomic status	Increased risk of chromosomally normal abortion among socially disadvantaged populations; results may be confounded by differences in ethnicity, patterns of medical care utilization, environmental exposures, etc
Previous abortion	Conflicting evidence regarding role of multiple, prior, induced abortions; may depend on method of termination; prior spontaneous losses increase risk of recurrence; repeat losses to the same woman tend to be chromosomally normal
Immunological factors	Increased risk of chromosomally normal losses; important etiological factor in recurrent abortion
Hormonal factors	Luteal phase defects implicated in recurrent abortion; in utero DES exposure associated with increased risk of spontaneous loss; no increased loss rate among users of oral contraceptives
Chronic diseases	Risk in diabetics relates to degree of glucose control; some studies show increased loss rates among untreated epileptics, others do not; systemic lupus erythematosus associated with increased risk; role of thyroid diseases remains unclear
Anatomic abnormalities	Uterine anomalies associated with increased risk of chromosomally normal loss; cervical incompetence increases risk of mid-trimester abortion; risk factors include prior cervical surgery (dilatation and curettage, amputation, conization), DES exposure, parity
Maternal fever	Increased risk of chromosomally normal abortion; difficult to separate role of fever from infection itself
Cigarette smoking	Modest dose-related effect on risk for chromosomally normal abortion; in one study, increased risk found only in socially disadvantaged women
Alcohol	Dose-related increase in risk of chromosomally normal loss; in one study, effect noted only in socially disadvantaged women
Irradiation	Possible association with aneuploid abortion (triploidy, possibly trisomy)

Sources: Modified from Kline, J. et al., *Conception to Birth: Epidemiology of Prenatal Development*, Oxford University Press, New York, 1989; From Shepard, T.H. et al., Developmental toxicology: Prenatal period, Chapter 4, in *Occupational and Environmental Reproductive Hazards: A Guide to Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993. With permission.

TABLE 22.21
Probabilities of Spontaneous Abortion

Time from Ovulation	Probability of Fetal Death in Gestation Interval (%)
1–6 days	54.6
7–13 days	24.7
14–20 days	8.2
3–5 weeks	7.6
6–9 weeks	6.5
10–13 weeks	4.4
14–17 weeks	1.3
18–21 weeks	0.8
22–25 weeks	0.3
26–29 weeks	0.3
30–33 weeks	0.3
34–37 weeks	0.3
38+ weeks	0.7

Sources: Modified from Kline, J. and Stein, Z., Very early pregnancy, in *Reproduction Toxicology*, Dixon, R.L. Ed., Raven Press, New York, p. 259, 1985; In McGuigan, M.A., Teratogenesis and reproductive toxicology, Chapter 16, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.

TABLE 22.22
Factors Known to Cause Fetal Growth Retardation in Humans

Maternal

Genetic
Stature
Maternal diseases (e.g., chronic pulmonary disease, sickle cell anemia)
Malnutrition
Hypoxia (high altitude)
Immunological factors
Metabolic diseases
Uterine anomalies
After induced abortion
Maternal addiction (e.g., heroin)
Smoking (nicotine?)
Alcoholism
Socioeconomic influences

Fetal

Genetic and chromosomal (e.g., trisomies 13, 15, and 21 and Turner's syndrome)
Congenital malformations (e.g., anencephaly and cardiac malformations) Rh hemolytic disease
Twin-to-twin transfusion
Endocrine disorders
Hydramnios
Multiple gestation (twins)
Infections (e.g., rubella, cytomegalovirus, syphilis, toxoplasmosis, malaria)
Nonionizing radiation
Certain drugs (e.g., aminopterin, busulfan)

Placental

Metabolic disturbances
"Placental insufficiency," postmature "aged" placenta, reduced uteroplacental circulation
Abnormal implantation (e.g., *placenta previa*)
Single umbilical artery
Velamentous insertion of umbilical cord
Circumvallate placenta
Abruptio placentae
Infarctions
Avascularity of chorionic villi
Hemangioma
Fibrinous exudation

Sources: Modified from Persaud, T.V.N., Teratogenic mechanisms, in *Advances in the Study of Birth Defects*, Vol. 1, University Park Press, Baltimore, MD, 1979; From Rousseaux, C.G. and Blakley, P.M., Fetus, Chapter 25, in *Handbook of Toxicologic Pathology*, Haschek, W.M. and Rousseaux, C.G. Eds., Academic Press, San Diego, CA, 1991. With permission.

TABLE 22.23
Frequency of Selected Adverse Pregnancy Outcomes in Humans

Event	Frequency per 100	Unit
Spontaneous abortion, 8–28 weeks	10–20	Pregnancies or women
Chromosomal anomalies in spontaneous abortions, 8–28 weeks	30–40	Spontaneous abortions
Chromosomal anomalies from amniocentesis	2	Amniocentesis specimens
Stillbirths	2–4	Stillbirths and live births
Low birth weight <2500 g	7	Live births
Major malformations	2–3	Live births
Chromosomal anomalies	0.2	Live births
Severe mental retardation	0.4	Children to 15 years of age

Sources: Modified from National Foundation/March of Dimes: Report of Panel II. Guidelines for reproductive studies in exposed human populations, In: *Guidelines for Studies of Human Populations Exposed to Mutagenic and Reproductive Hazards*, Bloom, A.D. Ed., The Foundation, New York, p. 37, 1981; From Manson, J.M. and Wise, L.D., Teratogens, Chapter 7, in *Cassarett and Doull’s Toxicology: The Basic Science of Poisons*, 4th edn., Amdur, M.O., Doull, J., and Klaassen, C.D. Eds., Pergamon Press, New York, 1991. With permission.

TABLE 22.24
Criteria for Recognizing a New Teratogen in Humans

- An abrupt increase in the frequency of a particular defect or association of defects (syndrome)
- Coincidence of this increase with a known environmental change, such as widespread use of a new drug
- Known exposure to the environmental change at a particular stage of gestation yielding a characteristically defective syndrome
- Absence of other factors common to all pregnancies yielding infants with the characteristic defect

Sources: Data from Wilson, J.G., *Fed. Proc.*, 36, 1698, 1977; Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5. With permission.

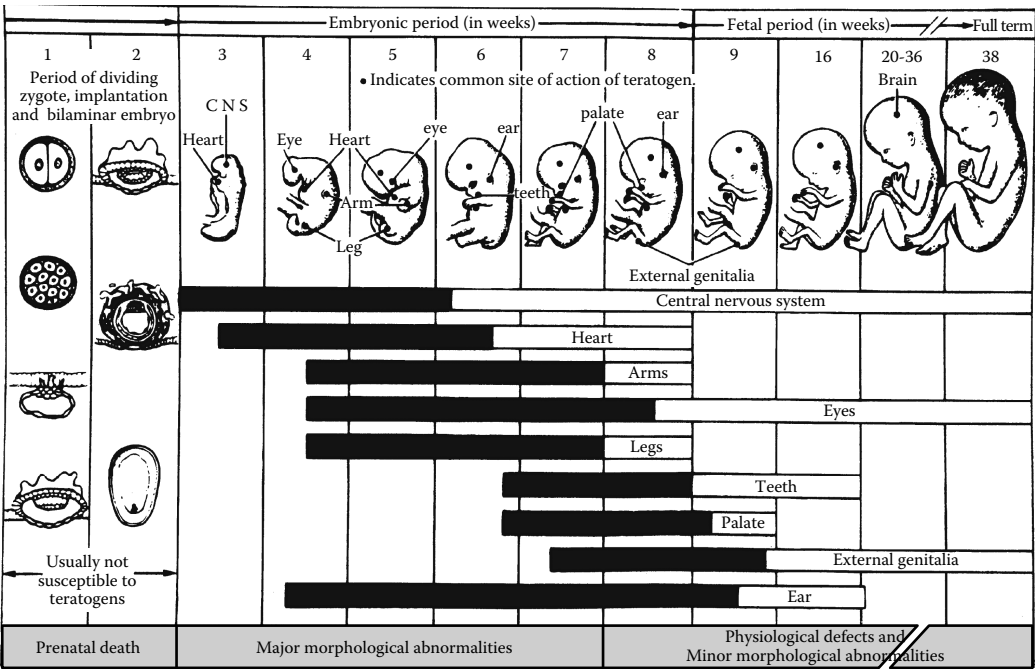


FIGURE 22.29 Schematic diagram of critical periods of human development. For organs and structures indicated within horizontal bars, the solid area represents periods of high sensitivity to teratogens; clear areas represent less sensitive periods. (From Moore, K.L., *The Developing Human: Clinically Oriented Embryology*, 5th edn., Saunders, Philadelphia, PA, 1993. With permission.)

TABLE 22.25
Causes of Malformation in Humans

Unknown	65%–70%
Genetic defects	20%
Drugs/environmental chemicals	4%–6%
Chromosomal abnormalities	3%–5%
Maternal infections	2%–3%
Maternal metabolic imbalances	1%–2%
Maternal reactions	<1%
Potentiative interactions	?

Sources: Modified from Wilson, J.G., *Fed. Proc.*, 36, 1698, 1977; From McGuigan, M.A., Teratogenesis and reproductive toxicology, Chapter 16, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J.B. and Krieger, G.R. Eds., Williams & Wilkins, Baltimore, MD, 1992. With permission.

TABLE 22.26
Incidence of Human Birth Defects per 1000

Defect	Spontaneous Abortuses	Elective Termination	Stillborn and Premature	Newborns
Neural tube defects	14	2.4	4.4	0.9
Spina bifida and cephalo	— ^c	—	—	0.9
Hydrocephalus	—	—	—	0.4
Heart malformations	—	—	—	3.0
Cleft palate	1.3	1.1	4.0	2.7
Cleft palate and cleft lip	6.9	3.2	8.0	0.4
Hypospadias ^a	—	—	—	5.1
Clubfoot	—	—	—	2.6
Cyclopia	2.7	2.1	—	0
Polydactyly	2.7	2.8	—	1.4
Sirenomelia ^b and caudal regression	4.2	—	—	0
Defects of intestines and/or trachea	—	—	—	0.5
Kidney inadequacy	—	—	—	0.1
Down's syndrome	—	—	—	0.8
Turner's syndrome	90	—	—	0.05
Total anomalies	200	—	90–140	20–30

Sources: Data from Shepard, T.H. et al., *Teratology*, 39, 325, 1989; Fantel, A.G. and Shepard, T.H., Morphological analysis of spontaneous abortuses, in *Spontaneous and Recurrent Abortion*, Bennett, M.J. and Edmons, D.K. Eds., Blackwell Publications, Oxford, U.K., p. 8, 1987; MMWR, Annual Summary, *Morb. Mortal. Wkly. Rep.*, 30(54), 108, 1981; Shepard, T.H. et al., Developmental toxicology: Prenatal period, Chapter 4, in *Occupational and Environmental Reproductive Hazards: A Guide to Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993; Crone, H.D., *Chemicals and Society: A Guide to the New Chemical Age*, Cambridge University Press, Cambridge, U.K., 1986, Chapter 7.

^a Displacement of opening of urinary tract from top of penis and genital organs in varying degrees of underdevelopment.

^b Union of the legs with partial or complete fusion of the feet.

^c No data found.

TABLE 22.27
Known Human Teratogens

Radiation		Drugs and Chemicals	
Therapeutic		Accutane (13-cis-retinoic acid)	Microprostil
Radioiodine		ACE inhibitors	Nor-progesterones (high doses)
Atomic weapons		Aminopterin	Penicillamine
Infections		Androgenic hormones	Phenobarbital
Varicella		Antithyroid drugs	Polychlorobiphenyls
Rubella		Aspirin	Primidone
Cytomegalovirus		Busulfan	Streptomycin
Herpes simplex virus		Carbamazepine	Tetracyclines
Toxoplasmosis		Chlorobiphenyls	Thalidomide
Venezuelan equine encephalitis virus		Cigarette smoking (nicotine)	Triamterene
Syphilis		Cocaine	Trimethadione
Maternal disorders		Coumarin anticoagulants	Trimethoprim
Hypo/hyper thyroidism		Cyclophosphamide	Valproic acid
Diabetes (insulin dependent)		DES	Vitamin D
Phenylketonuria		Diphenylhydantoin (phenytoin)	
Virilizing tumors, metabolic conditions		Fluconazole	
Alcoholism		Lithium	
Hyperthermia		Methimazole	
Hypertension		Methotrexate	
Rheumatic disease and congenital heart block		Methyl mercury	
Autoimmune disorders		Methylene blue	

Sources: Adapted from Manson, J.M. and Wise, L.D., Teratogens, Chapter 7, in *Cassarett and Doull's Toxicology: The Basic Science of Poisons*, 4th edn., Amdur, M.O., Doull, J., and Klaassen, C.D. Eds., Pergamon Press, New York, 1991; The Mountain States Genetic Foundation, *The Genetic Drift Newsletter*, Vol. 12, The Mountain States Genetic Foundation, 1995; Farrer, F., *SA Pharm. J.*, 10, 28, 2010.

TABLE 22.28
Etiologic Agents for Congenital Malformations in Humans and/or Domestic Animals^a

Agent	Effect
Alcohol	Pre- and postnatal growth retardation, MR, unusual facial features, congenital heart defects, urogenital defects, skeletal defects
Amantadine hydrochloride (±)	Congenital heart defect, pulmonary atresia
Aminopterin	Hydrocephaly, CP, meningocoele, meningocele, reduced derivatives of first bronchial arch
Anagryne (Lupins)	Crooked calf disease, scoliosis, arthrogryposis, CP
Anesthetics	Increased spontaneous abortions, CNS defects, musculoskeletal defects
Benztropine mesylate (±)	Left colon syndrome
Boric acid (±)	Increased risk for major malformation, especially cataracts
Bromide (±)	Short stature, small cranium, congenital heart disease
Busulfan (±)	CP, eye defects, generalized cytomegaly
Calcium carbonate	CNS defects
Carbon monoxide	CNS defects
Wild black cherry	Syringomyelia, rudimentary external genitalia, anal atresia, blindly ending colon (pigs)
Chlorambucil (±)	Renal agenesis
Chloroquine (±)	Congenital deafness, chorioretinitis, hemihypertrophy
Cigarette smoking	Increased spontaneous abortion, prematurity, IUGR
Clomiphene (±)	Anencephaly, microcephaly
Coniine (<i>Conium maculatum</i>)	Arthrogryposis, scoliosis
Copper deficiency	"Swayback," enzootic ataxia (sheep)
Coumarin derivatives	Nasal hypoplasia, calcific stippling of dicumarol, warfarin secondary epiphyses, hydrocephaly
Cyclopamine (<i>Veratrum californicum</i>)	Cyclopia, CP, cerebral defects
Cyclophosphamide (±)	Ectrodactyly, brachydactyly, flattened nasal bridge
Dextroamphetamine	Atrial and ventricular septal defect, biliary sulfate (±) atresia, facial clefts
Diabetes	Caudal regression syndrome, CP, defects of branchial arches
Diazepam	Facial clefts, CP
DES	Hypospadias, male and female pseudohermaphroditism, vaginal adenocarcinoma
Diphenylhydantoin	CL/CP, congenital heart disease, microcephaly, hypoplasia of nails and distal phalanges

TABLE 22.28 (continued)

Etiologic Agents for Congenital Malformations in Humans and/or Domestic Animals^a

Agent	Effect
Enovid-R (oral progestin) (±)	Female pseudohermaphroditism
17- α -Ethinyltestosterone	Female pseudohermaphroditism
Ethionamide (±)	Congenital heart defects, spina bifida, gastrointestinal atresia
Fasting, starvation	Hydrocephaly, meningomyelocele
Fluorine	Mottled tooth enamel
5-Fluorouracil (±)	Radial aplasia, imperforate anus, esophageal aplasia, hypoplasia of duodenum, lung, and aorta
Folic acid deficiency	Neural tube defects
Griseofulvin	CP (cats)
17-Hydroxyprogesterone (±)	Female pseudohermaphroditism
Hypertension	IUGR, microcephaly, patent ductus arteriosus, hypotonia of skeletal and gut musculature
Hyperthermia	Microcephaly, microphthalmia, anencephaly, spina bifida
Hypervitaminosis A	Ectopic ureter, CP, craniofacial defects, skeletal malformations
Hypoxia (±)	Decreased birth weight, patent ductus arteriosus (may be a postnatal effect)
Imipramine (±)	Limb reduction deformities
Indomethacin (±)	Pulmonary artery changes
Insulin (±)	Fetal deaths, multiple congenital anomalies
Iodine deficiency	Endemic cretinism, hyperthyroidism
Iodine excess	Congenital goiter, hypothyroidism
Isoniazid (±)	Increased risk for malformations
Isotretinoin	Hydrocephaly, micrognathia, low-set ears, microcephaly, microphthalmia, malformed skull, ventricular septal defect
Lathyrism (Lathyrus)	Poorly developed muscles and connective tissue, dissecting aneurysms of aorta, spinal malformations, CP (domestic animals)
Lead	Increased stillbirth and spontaneous abortion, MR
Lithium carbonate	Epstein's anomaly
Locoweed (<i>Astragalus</i> , <i>Oxytropis</i>)	Arthrogryposis
Lysergic acid (±)	Increased spontaneous abortions
Marijuana (±)	IUGR, developmental delays
Medroxyprogesterone (±)	Female pseudohermaphroditism, hypospadias
Meprobamate (±)	Congenital heart defect, increased malformation rate with no specific pattern
Mercury	Cerebral palsy, microcephaly, MR
Methallibure	Contractures of distal extremities, distorted mandible and cranial bones, dysplasia of renal cortex (pigs)
Methimazole (±)	Midline defect of scalp
Methotrexate	Absence of frontal bone, premature craniosynostosis, rib defects, ectrodactyly
Methyltestosterone	Female pseudohermaphroditism
Myasthenia gravis	Congenital contractures
Neguvon	Congenital tremors with hypoplasia of cerebellum (pigs)
Oral contraceptives (±)	Congenital heart defects, limb reduction deformities
Oxytetracycline	Stains deciduous tooth enamel
O-Penicillamine	Lax skin, inguinal hernia, flexion contractures of knee and hip
Phenothiazine (±)	Increased malformation rate
Phenylalanine excess (maternal PKU)	Microcephaly, IUGR, congenital heart defects, dislocation of hips, strabismus
Phenylpropanolamine	Eye and ear defects, hypospadias
Phenobarbital (±)	Fetal hydantoin-like syndrome
Polychlorinated biphenyls	Cola-colored babies, IUGR, exophthalmos, staining of skin, and gums
Pregnancy test tablets (±)	Neural tube defects, congenital heart defects
Primidone (±)	Low nasal bridge, ocular hypertelorism, pulmonic stenosis
Progesterone (±)	Hypospadias
Propylthiouracil	Congenital goiter
Quinine (±)	Congenital deafness, hydrocephaly, limb, facial, gastrointestinal, and urogenital defects
Reserpine (±)	Congenital lung cysts
Rheumatic disease of mother (especially systemic lupus erythematosus)	Congenital heart block
Organic solvents (±)	Neural tube defects, hydrocephaly, congenital heart defects, talipes
Streptomycin (±)	Congenital deafness
Testosterone	Female pseudohermaphroditism
Tetracycline	Staining of enamel of deciduous or permanent teeth
Thalidomide	Limb reduction anomalies, polydactyly, ear defects, facial hemangioma, esophageal or duodenal atresia, tetralogy of Fallot, renal agenesis

(continued)

TABLE 22.28 (continued)

Etiologic Agents for Congenital Malformations in Humans and/or Domestic Animals^a

Agent	Effect
Tobacco stalk	Arthrogryposis (pigs)
Trimethadione	Cp, cardiac defects, V-shaped eyebrows, developmental delays, low-set ears, irregular teeth
Valproic acid	Microcephaly, facial dysmorphology, congenital heart defect, neural tube defect
Virilizing tumor	Female pseudohermaphroditism
Vitamin D excess (±)	Supravalvular aortic stenosis, elfin faces, MR
X-irradiation	Microcephaly, MR, hydrocephaly, CP, hypospadias, hypoplastic genitalia, IUGR, microphthalmia, cataracts, strabismus, retinal degeneration and pigment changes, skeletal defects
Zinc deficiency	Anencephaly, achondrogenesis

Source: From Rousseaux, C.G. and Blakley, P.M., Fetus, Chapter 25, in *Handbook of Toxicologic Pathology*, Haschek, W.M., and Rousseaux, C.G. Eds., Academic Press, San Diego, CA, 1991. With permission.

^a MR, mental retardation; CNS, central nervous system; CL/CP, cleft lip/cleft palate; IUGR, intrauterine growth retardation; (±), questionable association.

RELATIVE RISK TABLES (TABLES 22.29 THROUGH 22.32)

As an aid for making risk comparisons, this section contains reference risk values for death from various types of cancer and for factors believed to cause cancer. In addition, risk values for various occupations, lifestyles, and activities are also presented. For Tables 22.29 through 22.32, refer to the cited source for information on how these values were determined and the uncertainty associated with them.

In the risk assessment process, risk is expressed in quantitative terms that form the basis for risk management decisions. Generally, carcinogens are regulated to “safe” exposure levels based on risk levels considered “acceptable” to society. Regulatory agencies such as the US Environmental Protection Agency consider a risk of 1×10^{-6} or one in one million to be acceptable. However, this number can vary from 10^{-4} to 10^{-7} depending on the substance of concern and the circumstances of the exposure.

Quantitative risk is a difficult concept for the general public to comprehend. It is often necessary to put risk in perspective to convey the magnitude of an unfamiliar risk. A risk of one in one million is easier to relate to when it is compared to a familiar reference point, for example, the risk of death from driving an automobile.

Risk comparisons are useful in two other ways: to compare the risks of alternative options and to gauge the importance of different causes of the same hazard.⁵⁴ In the former case, an example would be a risk comparison between a new and existing chemical to show the value of introducing the new product. An example of the latter type of comparison would be the ranking of the risk associated with various environmental factors suspected of causing of lung cancer to determine where remediation efforts would have the greatest impact.

Risk comparisons should not be used to attempt to trivialize or make a risk more acceptable. The acceptability of risk is a matter of perception based on numerous subjective qualitative factors related to a hazard’s perceived “dreadfulness” and how

well it is understood (see Figure 22.10). Risks that are similar quantitatively, but differ qualitatively, will not be perceived as equally acceptable. Risks perceived as voluntary are considered more acceptable than risks perceived as involuntary. For example, the risk of developing cancer from smoking may be viewed as more acceptable than the risk of developing lung cancer from an air pollutant, even though the risk from smoking may be several orders of magnitude greater.

Where possible, risks of similar qualitative characteristics should be compared. Avoid “apples and oranges” comparisons such as comparing the uncontrollable to the controllable, technological to natural, involuntary to voluntary. The reader is referred to the National Research Council’s publication entitled *Improving Risk Communication*⁵⁴ for a detailed discussion of the uses, misuses, and pitfalls associated with risk comparisons.

TABLE 22.29
Lifetime Risk and Annual Average Risk of Death
from Cancer in the United States^a

Type	Lifetime Risk	Average Annual Risk
All cancers	0.20	2.8×10^{-3}
Buccal cavity, pharynx, respiratory	0.050	7.2×10^{-4}
Digestive organs and peritoneum	0.053	7.5×10^{-4}
Bone, connective tissue, skin, breast	0.022	3.1×10^{-4}
Genital organs	0.022	3.2×10^{-4}
Urinary tract	0.008	1.2×10^{-4}
Leukemia, other blood, and lymph	0.018	2.6×10^{-4}
Other	0.019	2.7×10^{-4}

Source: Crouch, E.A.C. and Wilson, R., Inter-risk comparisons, Chapter 7, in *Assessment and Management of Chemical Risks*, Rodricks, J.V. and Tardiff, R.G. Eds., American Chemical Society, Washington, DC, 1984. With permission.

^a The uncertainty in all these values is about 20%.

TABLE 22.30
Cancer Risks from Radiation Exposures

Type	Average Annual Risk
Natural background (average United States, sea level)	2×10^{-5}
US average medical diagnostic x-ray	2×10^{-5}
Excess due to living in masonry building rather than wood	5×10^{-6}
Cosmic rays	
Airline pilot (50 h/month at 12 km altitude)	4×10^{-5}
One transcontinental round trip by air per year	1×10^{-6}
Frequent airline passenger (4 h/week)	1×10^{-5}
Living in Colorado compared with New York	8×10^{-6}
Camping at 15,000 ft for 4 month/year	2×10^{-6}

Source: Crouch, E.A.C. and Wilson, R., Inter-risk comparisons, Chapter 7, in *Assessment and Management of Chemical Risks*, Rodricks, J.V. and Tardiff, R.G. Eds., American Chemical Society, Washington, DC, 1984. With permission.

TABLE 22.31
Everyday Cancer Risks from Common Carcinogens

Action	Average Annual Risk	Uncertainty
One 12.5 oz diet soda daily (saccharin)	1×10^{-5}	Factor of ~ 10
Average personal saccharin consumption	2×10^{-6}	Factor of ~ 10
4 tablespoon peanut butter/day (aflatoxins)	8×10^{-6}	Factor of ~ 10
1 pint of milk/day (aflatoxins)	2×10^{-6}	Factor of ~ 10
Miami/New Orleans drinking water	1×10^{-6}	Factor of ~ 10
1/2 lb charcoal broiled steak/week (cancer only; heart attack, etc., extra)	3×10^{-7}	Factor of ~ 10
Average smoker (cancer only)	1.2×10^{-3}	Factor of 3 (human data)
(All effects)	3×10^{-3}	Factor of 3 (human data)
Person sharing room with smoker	1×10^{-5}	Factor of ~ 10
Air pollution (polycyclic organics)	1.5×10^{-5}	Factor of ~ 10

Source: Crouch, E.A.C. and Wilson, R., Inter-risk comparisons, Chapter 7, in *Assessment and Management of Chemical Risks*, Rodricks, J.V. and Tardiff, R.G. Eds., American Chemical Society, Washington, DC, 1984. With permission.

TABLE 22.32
Risk of Death in the United States

Industry Group	Annual Risk of Death
Trade	5.3×10^{-5}
Manufacturing	8.2×10^{-5}
Service and government	1.0×10^{-4}
Transport and public utilities	3.7×10^{-4}
Agriculture	6.0×10^{-4}
Construction	6.1×10^{-4}
Mining and quarrying	9.5×10^{-4}
More Finely Divided Grouping	
Farming	3.6×10^{-4}
Stone quarries and mills	5.9×10^{-4}
Police officers (in line of duty)	2.2×10^{-4}
Railroad employee	2.4×10^{-4}
Steelworker (accident only)	2.8×10^{-4}
Firefighter	8.0×10^{-4}
Accident	
Motor vehicle	2.4×10^{-4}
All home accidents	1.1×10^{-4}
Fall	6.2×10^{-5}
Drowning	3.6×10^{-5}
Fire	2.8×10^{-5}
Inhalation/ingestion of objects	1.5×10^{-5}
Accidental poisoning	1.4×10^{-5}
Firearms (accidents)	1.0×10^{-5}
Electrocution	5.3×10^{-6}
Tornado	6×10^{-7}
Flood	6×10^{-7}
Lightning	5×10^{-7}
Tropical cyclone/hurricane	3×10^{-7}
Bite/sting	2×10^{-7}
Sport	
Professional stunting	$<1 \times 10^{-2}$
Air show/air racing and acrobatics	5×10^{-3}
Flying amateur/home built aircraft	3×10^{-3}
Sport parachuting	2×10^{-3}
Professional aerial acrobatics	$<2 \times 10^{-4}$
Hang gliding	8×10^{-4}
Mountaineering	6×10^{-4}
Glider flying	4×10^{-4}
Scuba diving	4×10^{-4}
Spelunking	$<1 \times 10^{-4}$
Boating	5×10^{-5}
College football	3×10^{-5}
Hunting	3×10^{-5}
Swimming	3×10^{-5}
Ski racing	2×10^{-5}

Source: Modified from Crouch, E.A.C. and Wilson, R., Inter-risk comparisons, Chapter 7, in *Assessment and Management of Chemical Risks*, Rodricks, J.V. and Tardiff, R.G. Eds., American Chemical Society, Washington, DC, 1984. With permission.

STANDARD RISK ASSESSMENT REFERENCE VALUES* (FIGURES 22.30 AND 22.31; TABLES 22.33 THROUGH 22.50)

The risk assessment process involves extrapolation of dose–response data from animals to humans and quantitative estimates of human exposure. For this to be accomplished, a detailed understanding of interspecies differences, population diversity, and environmental factors is critical. Comparative quantitative morphological, physiological, and biochemical information is required for different species. The heterogeneity of exposed populations with respect to such factors as age, lifestyle, and activity patterns must be characterized. Measurements of environmental factors affecting the distribution and fate of the substance from source to exposed human must be obtained (see Figure 22.7).

Accurate data for the type of information described earlier are not always available. Although exposure determinations are best made at the site where people live and work, problems of sampling, unequal distribution of exposure, fluctuations in environmental conditions, and many other confounding factors make accurate representative measurements difficult to obtain. Characterizing the diversity and lifestyles of a population becomes more difficult as both the size of the population and the area of the exposure increase. The accuracy and relevance of limited data developed for diverse populations spread out over vast areas is questionable. The cost of characterizing such a population becomes prohibitive. For this reason, estimates, rather than actual measurements, are most often used in risk assessment. However, the use of standard values of this sort introduces a source of uncertainty regarding the relevance of the values to the exposed population.

Risk assessment usually relies on a combination of measured and estimated values. The more accurate and representative the measured values, the more realistic will be the risk assessment because the quality of the conclusions will only be as good as the information on which the assessment is based.

This section presents standard comparative reference values useful for extrapolating animal exposures to humans. Also provided are standard values related to human lifestyles and activity patterns needed for making exposure estimates. Reference values for environmental factors affecting fate and distribution are beyond the scope of this book.

The reference values presented may differ from values available from other sources and should not be considered to be more valid than those that can be obtained elsewhere. The reference values presented in this section, however, reasonably represent the available data. The reader is cautioned that these values may not be reliable for all situations. To the extent possible, the use of standard values in risk assessment should be avoided where accurate and reliable measurements can be made from the real world.

TABLE 22.33
The Duration of Studies in Experimental Animals and Time Equivalents in Humans

Species	Duration of Study In Months				
	1	3	6	12	24
	Percent of Life Span				
Rat	4.1	12	25	49	99
Rabbit	1.5	4.5	9	18	36
Dog	0.82	2.5	4.9	9.8	20
Pig	0.82	2.5	4.9	9.8	20
Monkey	0.55	1.6	3.3	6.6	13
	Human Equivalents (In Months)				
Rat	34	101	202	404	808
Rabbit	12	36	72	145	289
Dog	6.5	20	40	81	162
Pig	6.5	20	40	81	162
Monkey	4.5	13	27	61	107

Sources: Modified from Paget, G.E. Ed., *Methods in Toxicology*, Blackwell Scientific Publishers, Oxford, U.K., p. 49, 1970; From Ecobichon, D.J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 4. With permission.

* See Appendix B, "Tables of Comparative Anatomical, Physiological, and Biochemical Data" section for additional information.

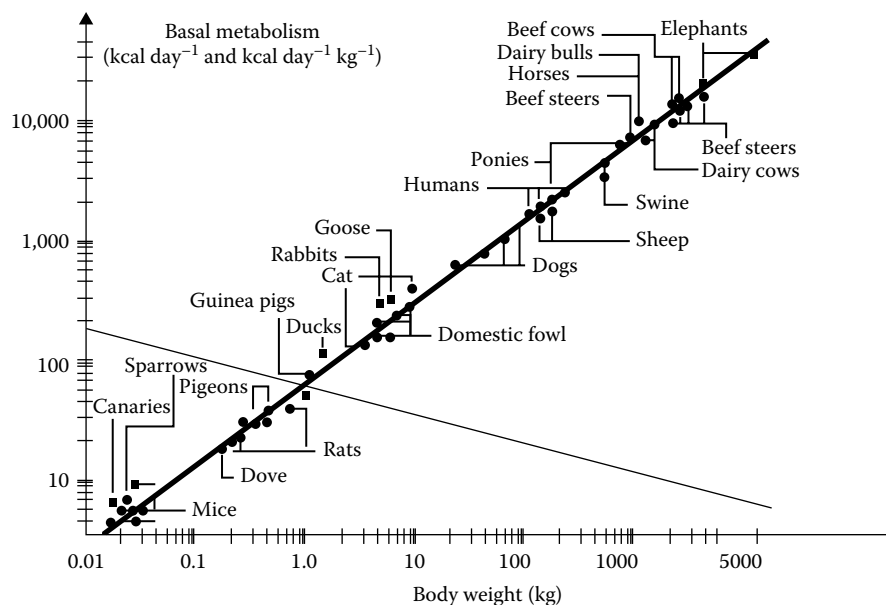


FIGURE 22.30 Interspecies extrapolation of metabolic rate showing that metabolic rates increase with the three-fourths power of body weight ($BW^{0.75}$) for species covering a broad size range. (Reprinted from Kleiber, M., *The Fire of Life: An Introduction to Animal Energetics*, John Wiley & Sons, New York, 1961. With permission from the American Physiological Society.)



FIGURE 22.31 Schematic representation of development changes in hepatic metabolism and renal elimination. Prenatally and at birth, function is less than 50% of adult values. During infancy and early childhood, function increases beyond adult values. During puberty, values decline toward typical adult levels. These changes are important to consider in a risk assessment because they can have significant impact on the risk of exposure to substances that undergo biotransformation and/or renal elimination. (From Mortensen, M.E., *Mercury toxicity in children*, in *Similarities and Differences between Children and Adults: Implications for Risk Assessment*, Guzelian, P.S., Henry, C.J., and Olin, S.S. Eds., ISLI Press, Washington, DC, p. 204, 1992. Copyright 1992. With permission from the International Lifesciences Institute. All rights reserved.)

TABLE 22.34
Summary of Drug Absorption in Neonates, Infants, and Children

	Neonate	Infants	Children
Physiological Alteration			
Gastric emptying time	Irregular	Increased	Slightly increased
Gastric pH	>5	4 to 2	Normal (2–3)
Intestinal motility	Reduced	Increased	Slightly increased
Intestinal surface area	Reduced	Near adult	Adult pattern
Microbial colonization	Reduced	Near adult	Adult pattern
Biliary function	Immature	Near adult	Adult pattern
Muscular blood flow	Reduced	Increased	Adult pattern
Skin permeability	Increased	Increased	Near adult pattern
Possible Pharmacokinetic Consequences			
Oral absorption	Erratic— reduced	Increased rate	Near adult pattern
I.M. absorption	Variable	Increased	Adult pattern
Percutaneous absorption	Increased	Increased	Near adult pattern
Rectal absorption	Very efficient	Efficient	Near adult pattern
Pre-systemic clearance	<adult	>adult	>adult

Sources: From Ritschel, W.A. and Kearns, G.L., Table 24.1 “Summary of Drug Absorption in Neonates, Infants and Children,” *Handbook of Basic Pharmacokinetics*, 5th edn., American Pharmaceutical Association, Washington, DC, p. 307, 1999. Copyright 1999. With permission of the American Pharmaceutical Association; Originally adapted from Morselli, P.L., *Clin. Pharmacokin.*, 17(Suppl 1), 13, 1989.

Note: Direction of alteration given relative to expected normal adult pattern. Data contained in the table reflect developmental differences that might be expected in healthy pediatric patients. Certain conditions/disease states might modify the function and/or structure of the absorptive surface area, GI motility, and/or systemic blood flow impacting on the rate or extent of absorption. Generally, neonate \leq 1 month of age, infants = 1–24 months of age, children = 2–12 years of age. As the age limits defining these developmental stages are somewhat arbitrary, some overlap in the functional capacity between these stages should be expected. Because physiological development is a dynamic process, it should be kept in mind that functional changes occur incrementally over time and do not abruptly change from one age group to another.

TABLE 22.35
Plasma Protein Binding and Drug Distribution in Neonates, Infants, and Children

	Neonate	Infants	Children
Physiological Alteration			
Plasma albumin	Reduced	Near normal	Near adult pattern
Fetal albumin	Present	Absent	Absent
Total proteins	Reduced	Decreased	Near adult pattern
Total globulins	Reduced	Decreased	Near adult pattern
Serum bilirubin	Increased	Normal	Normal adult pattern
Serum free fatty acids	Increased	Normal	Normal adult pattern
Blood pH	7.1–7.3	7.4 (normal)	7.4 (normal)
Adipose tissue	Scarce (\uparrow CNS)	Reduced	Generally reduced
Total body water	Increased	Increased	Near adult pattern
Extracellular water	Increased	Increased	Near adult pattern
Endogenous maternal Substances (Ligands)	Present	Absent	Absent
Possible Pharmacokinetic Consequences			
Free fraction	Increased	Increased	Slightly increased
Apparent volume of distribution			
Hydrophilic drugs	Increased	Increased	Slightly increased
Hydrophobic drugs	Reduced	Reduced	Slightly decreased
Tissue/plasma ratio	Increased	Increased	Slightly increased

Sources: From Ritschel, W.A. and Kearns, G.L., Table 24.2 “Plasma Protein Binding and Drug Distribution,” *Handbook of Basic Pharmacokinetics*, 5th edn., American Pharmaceutical Association, Washington, DC, p. 309, 1999. Copyright 1999. With permission by the American Pharmaceutical Association; Originally adapted from Morselli, P.L., *Clin. Pharmacokin.*, 17(Suppl 1), 13, 1989.

Note: Direction of alteration given relative to expected normal adult pattern. Generally, neonate \leq 1 month of age, infants = 1–24 months of age, children = 2–12 years of age. As the age limits defining these developmental stages are somewhat arbitrary, some overlap in the functional capacity between these stages should be expected. Because physiological development is a dynamic process, it should be kept in mind that functional changes occur incrementally over time and do not abruptly change from one age group to another.

TABLE 22.36
Renal Function in the Neonate, Infant, and Child

	Neonate	Infants	Children
Physiological Alteration			
Kidney/body weight ratio	Increased	Increased	Near adult values
Glomerular filtration rate	Reduced	Normal (by 12 mo)	Normal adult values
Active tubular secretion	Reduced	Near normal	Normal adult values ^a
Active tubular reabsorption	Reduced	Near normal	Normal adult values
Proteins present in urine	Present (30%)	Low to absent	Normally absent
Urinary acidification capacity	Low	Normal (by 1 month)	Normal adult activity
Urine output (mL/h/kg)	3–6	2–4	1–3
Urine concentrating capacity	Reduced	Near normal	Normal adult values
Possible Pharmacokinetic Consequences			
Active drug excretion	Reduced	Near normal	Normal adult pattern
Passive drug excretion	Reduced to		
	Increased	Increased	Normal adult pattern
Excretion of basic drugs	Increased	Increased	Near normal

Sources: Ritschel, W.A. and Kearns, G.L., Table 24.6 “Renal Function in the Neonate, Infant and Child,” *Handbook of Basic Pharmacokinetics*, 5th edn., American Pharmaceutical Association, Washington, DC, 1999. Copyright 1999. With permission of the American Pharmaceutical Association; Originally adapted from Morselli, P.L., *Clin. Pharmacokinetics*, 17(Suppl 1), 13, 1989.

Note: Direction of alteration given relative to expected normal adult patterns. Generally, neonate \leq 1 month of age, infants = 1–24 months of age, children = 2–12 years of age. As the age limits defining these developmental stages are somewhat arbitrary, some overlap in the functional capacity between these stages should be expected. Because physiological development is a dynamic process, it should be kept in mind that functional changes occur incrementally over time and do not abruptly change from one age group to another.

^a Denotes slight increase in excretion rate for basic compounds.

TABLE 22.37
Drug Metabolism in the Neonate, Infant, and Child

	Neonate	Infants	Children
Physiological Alteration			
Liver/body weight ratio	Increased	Increased	Slightly increased
Cytochromes P450 activity	Reduced	Increased	Slightly increased
Blood esterase activity	Reduced	Normal (by 12 months)	Adult pattern
Hepatic blood flow	Reduced	Increased	Near adult pattern
Phase II enzyme activity	Reduced	Increased	Near adult pattern
Possible Pharmacokinetic Consequences			
Metabolic rates	Reduced	Increased	Near adult pattern ^a
Pre-systemic clearance	Reduced	Increased	Near adult pattern
Total body clearance	Reduced	Increased	Near adult pattern ^a
Inducibility of enzymes	More evident	Slightly increased	Near adult pattern ^a

Sources: Ritschel, W.A. and Kearns, G.L., Table 24.5 “Drug Metabolism in the Neonate, Infant and Child,” *Handbook of Basic Pharmacokinetics*, 5th edn., American Pharmaceutical Association, Washington, DC, Copyright 1999. With permission of the American Pharmaceutical Association, p. 314; Originally adapted from Morselli, P.L., *Clin. Pharmacokinetics*, 17(Suppl 1), 13, 1989.

Note: Direction of alteration given relative to expected normal adult patterns. Generally, neonate \leq 1 month of age, infants = 1–24 months of age, children = 2–12 years of age. As the age limits defining these developmental stages are somewhat arbitrary, some overlap in the functional capacity between these stages should be expected. Because physiological development is a dynamic process, it should be kept in mind that functional changes occur incrementally over time and do not abruptly change from one age group to another.

^a Denotes assumption of adult pattern of activity after the conclusion of puberty. The activity of all drug-metabolizing enzymes is generally higher before vs. after puberty.

TABLE 22.38

Developmental Patterns for the Ontogeny of Important Drug-Metabolizing Enzymes in Man

Enzyme(s)	Known Developmental Pattern
Phase I Enzymes	
CYP2D6	Low to absent in fetal liver but present at 1 week of age. Poor activity (i.e., 20% of adult) by 1 month. Adult competence by 3–5 years of age
CYP2C19, CYP2C9	Apparently absent in fetal liver. Low activity in first 2–4 weeks of life with adult activity reached by approximately 6 months. Activity may exceed adult levels during childhood and declines to adult levels after conclusion of puberty
CYP1A2	Not present in appreciable levels in human fetal liver. Adult levels reached by approximately 4 months and exceeded in children at 1–2 years of age. Adult activity reached after puberty
CYP3A7	Fetal form of CYP3A that is functionally active (and inducible) during gestation. Virtually disappears by 1–4 weeks of postnatal when CYP3A4 activity predominates but remains present in approximately 5% of individuals
CYP3A4	Extremely low activity at birth reaching approximately 30%–40% of adult activity by 1 month and full adult activity by 6 months. May exceed adult activity between 1–4 years of age, decreasing to adult levels after puberty
Phase II Enzymes	
NAT2	Some fetal activity by 16 weeks gestation. Poor activity between birth and 2 months of age. Adult phenotype distribution reached by 4–6 months with adult activity reached by 1–3 years
TPMT	Fetal levels approximately 30% of adult values. In newborns, activity is approximately 50% higher than adults with phenotype distribution that approximates adults. Exception is Korean children where adult activity is seen by 7–9 years of age
UGT	Ontogeny is isoform specific. In general, adult activity is reached by 6 to 24 months of age
ST	Ontogeny is isoform specific and appears more rapid than that for UGT. Activity for some isoforms may exceed adult levels during infancy and early childhood

Sources: From Ritschel, W.A. and Kearns, G.L., Table 24.4 “Developmental Patterns for the Ontogeny of Important Drug Metabolizing Enzymes in Man,” *Handbook of Basic Pharmacokinetics*, 5th edn., American Pharmaceutical Association, Washington, DC, p. 3122, 1999. Copyright 1999. With permission of the American Pharmaceutical Association; Originally adapted from Leeder, J.S. and Kearns, G.L., *Pediatr. Clin. North Am.*, 44, 55, 1997. Abbreviations include: CYP, cytochrome P450; NAT2, N-acetyltransferase-2; TPMT, thiopurine methyltransferase; UGT, glucuronosyltransferase and ST, sulfotransferase.

TABLE 22.39

Comparative Mammalian Reference Values for Relative Dose Calculations

Species	Average Life Span (Years)	Body Weight (kg)	Food Consumption (g/Day)	Food Consumption Factor ^a	Water Consumption (mL/Day)	Inhalation Rate (m ³ /Day)
Human	70	70	2000	0.028	1400	20
Mouse	1.5–2	0.03	4	0.13	6	0.052
Rat	2	0.35	18	0.05	50	0.29
Hamster	2.4	0.14	12	0.083	27	0.13
Guinea pig	4.5	0.84	34	0.040	200	0.40
Rabbit	7.8	3.8	186	0.049	410	2
Cat	17	3	90	0.030	220	1.2
Dog	12	12.7	318	0.025	610	4.3
Monkey (Rhesus)	18	8	320	0.040	530	5.4

Source: Modified from U.S. Environmental Protection Agency, *Development of Statistical Distributions or Ranges of Standard Factors Used in Exposure Assessments*, Office of Health and Environmental Assessments, EPA No. 600/8-85/010, NTIS, PB85-242667, 1985.

^a Fraction of body weight consumed per day as food.

TABLE 22.40
Reference Comparative Physiological Values^{a,b}

Parameter	Mouse	Rat	Human
Tissue perfusion (% of Cardiac Output)			
Brain	7.5 (2.0–13.0)	1.2	14.0 (13.0–15.0)
Heart	4.4 (2.8–6.0)	2.9	3.3 (2.6–4.0)
Kidney	24.8 (14.6–35.0)	17.8	22.0
Liver (total)	21.0	18.6 (17.0–26.0)	26.5 (26.0–27.0)
Liver (arterial only)	8.4	6.7	— ^c
Viscera	30.3	26.3	30.0
Adipose tissue	— ^c	4.5 (4.0–5.0)	4.7 (4.5–5.0)
Tissue volume (% of Body Weight)			
Heart	0.4	0.5	0.6
Kidney	1.5	0.9 (0.9–1.0)	1.1 (0.4–1.5)
Liver	5.0 (4.0–5.9)	4.0 (3.7–4.2)	3.0 (2.4–4.0)
GI tract	6.8	4.3 (3.0–5.5)	3.8 (3.0–4.5)
Fat	7.6 (4.0–9.8)	8.4 (7.0–9.0)	15.5 (9.0–23.1)
Blood	7.6	7.2 (4.9–9.0)	7.2
Muscle	59.0 (45.0–73.0)	59.0 (50.0–73.0)	52.4 (43.4–73.0)
Skin	14.5	16.0	4.3
Marrow	2.7	— ^c	2.5 (2.1–2.8)
Skeletal tissue	9.0	— ^c	— ^c
Cardiac Output			
Absolute (L/min)	0.0129 (0.110–0.160)	0.1066 (0.0730–0.1340)	5.59 (4.60–6.49)
Relative (L/min · kg)	0.535 (0.440–0.711)	0.327 (0.248–0.646)	0.080 — ^c
Alveolar Ventilation (L/min)			
	0.026 (0.012–0.039)	0.080 (0.075–0.085)	4.6 (4.0–5.8)
Minute Volume			
Absolute (L/min)	0.038 (0.024–0.052)	0.169 (0.057–0.336)	7.4 (6.0–9.0)
Relative (L/min · kg)	1.533 (1.239–1.925)	0.780 (0.142–2.054)	0.089 (0.014–0.127)
Respiratory Frequency (breaths/min)			
	171 (100–213)	117 (60–153)	14 (10–16)

Source: Data derived from U.S. Environmental Protection Agency, *Reference Physiological Parameters in Pharmacokinetic Modeling*, Arms, A.D. and Travis, C.C. Eds., Office of Risk Analysis, EPA No., 600/6-88/004, 1988.

^a Mean of reported values. Brackets contain range of reported values from which mean was calculated. Absence of range indicates value was from a single report.

^b Values presented are for unanesthetized animals.

^c No data found.

TABLE 22.41
Body Fluid Volumes for Men and Women

Parameter	Adult Male ^a		Adult Female ^b	
	Volume (L)	% of Body Weight	Volume (L)	% of Body Weight
Total body water	45.0	60	33.0	55
Extracellular water	11.25	15	9.0	15
Intracellular water	33.75	45	24.0	40
Total blood volume	5.4	7.2	4.3	7.2
Plasma volume	3.0	—	2.6	—
Erythrocyte volume	2.4	—	1.7	—

Source: Adapted from Plowchalk, D. et al., Comparative approach to toxicokinetics, In: *Occupational and Environmental Reproductive Hazards, A Guide for Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993, Chapter 3.

^a Volumes calculated for an adult male with a body weight of 75 kg and a hematocrit of 45%.

^b Volumes calculated for an adult female with a body weight of 60 kg and a hematocrit of 40%.

TABLE 22.42
Comparative Mammalian Organ Weights (g/100 g Body Weight)

Species	Brain	Heart	Adrenals ^a	Kidneys ^a	Lungs	Liver	Spleen	Testes ^a
Human	1.96	0.42	0.02	0.41	0.73	2.30	0.25	0.04
Mouse	1.35	0.68	0.02	2.60	0.66	5.29	0.32	0.62
Rat	0.46	0.32	0.01	0.70	0.40	3.10	0.20	0.92
Monkey (Rhesus)	2.78	0.38	0.02	0.54	1.89	2.09	0.14	0.03
Dog	0.59	0.85	0.01	0.30	0.94	2.94	0.45	0.15
Rabbit	0.40	0.35	0.02	0.70	0.53	3.19	0.04	0.13
Hamster	0.88	0.47	0.02	0.53	0.46	5.16	—	—
Guinea pig	1.33	0.53	0.07	1.17	1.18	5.14	0.21	0.65
Cat	0.77	0.45	0.02	1.07	1.04	2.59	0.29	0.07

^a Combined paired weight.

TABLE 22.43
Typical Human Exposure Values Useful in Risk Assessments

Body weight	
Young child (1–3 year)	13 kg
Older child (5 year)	20 kg
Typical adult	80 kg
(see Table 22.44 for more specific children's weight relative to age)	
Lifespan	
Male	75 year
Female	80 year
Inhalation rate	
Typical adult	16 m ³ /day
Child (<1 year)	4.0 m ³ /day
Child (1–12 year)	9.0 m ³ /day
Industrial worker (8 h work shift)	10 m ³ /shift
Industrial worker (24 h total)	20 m ³ /day
Reasonable worst case	30 m ³ /day
(see Table 22.50 for inhalation rates relative to age and degree of activity)	
Drinking water ingestion rate	
Adult (average)	1.2 L/day (16 mL/kg-day)
Adult (90th percentile)	3.1 L/day (42 mL/kg-day)
Infant (<1 year)	0.5 L/day
Food consumption rate (adults)	
Total average meat intake	1.8 g/kg-day
Total average vegetable intake	2.5 g/kg-day
Total average fruit intake	1.1 g/kg-day
Total average dairy intake	3.5 g/kg-day
Total average dairy intake (child 1–3 year)	43.2 g/kg-day
Total grain intake	2.2 g/kg-day
Total average fish intake	0.62 g/kg-day
Adult total food intake	2000 g/day
Breast milk intake rate	
Average (birth–6 months)	657 mL/day
Upper percentile	977 mL/day
Exposed skin surface	
Typical adult	0.20 m ²
Reasonable worst case	0.53 m ²
Swimming or bathing (average)	
Male	1.94 m ²
Female	1.69 m ²
(see Tables 22.46 through 22.48 for more specific information relative to age and body parts)	
Soil ingestion rate (soil + dust)	
Children	
Average	100 mg/day
Upper percentile	200 mg/day
Pica child (soil)	1000 mg/day
Adult (average)	50 mg/day
Activities (also Table 22.49)	
a. Showering (typically one event/day)	
Average	10 min/day
95th percentile	35 min/day
(a 5 min shower is estimated to use 40 gallons of water)	
b. Bathing (typically one event/day)	
Median	20 min/event
90th percentile	45 min/event

(continued)

TABLE 22.43 (continued)
Typical Human Exposure Values Useful in Risk Assessments

c. Time indoors	
All ages	20 h/day
Average residence volume	492 m ³ (0.45 air changes/h)
d. Time outdoors	
Children (ages 3–6 months)	0.4 h/day
Children (ages 6–12 months)	2.3 h/day
Children	5 h/day
Adults	1.5 h/day

Sources: U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989; U. S. Environmental Protection Agency, *Exposure Factors Handbook*, Wood, P., Phillips, L., Adenuga, A., Koontz, M., Rector, H., Wilkes, C., and Wilson, M. Eds., National Center for Environmental Assessment, EPA/ 600/p-95/002Fa, 1997; U. S. Environmental Protection Agency, *Exposure Factors Handbook: 2011 Edition*, National Center for Environmental Assessment, Washington, DC, EPA/ 600/R-09/052F, 2011.

TABLE 22.44
Mean Body Weight (kg) of Children by Age

Age (Years)	Boys	Girls	Both
1 to <3	11.9	11.2	11.6
3–5	17.6	17.1	17.4
6–8	25.3	24.6	25.0
9–11	35.7	36.2	36.0
12–14	50.5	50.7	50.6
15–17	64.9	57.4	61.2

Source: Modified from U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989.

TABLE 22.45
Constants for Estimating Surface Area of Mammals^a

Species	Constant (K)
Rat	9.6
Mouse	9.0
Rabbit	10.0
Guinea pig	9.0
Monkey	11.8
Dog	11.0
Cat	8.7

Source: Data derived from Spector, W.S. Ed., *Handbook of Biological Data*, W.B. Saunders, Philadelphia, PA, p. 175, 1956.

^a $A = KW^{2/3}$ where A = surface area (cm²); K = constant; W = body weight (g).

TABLE 22.46
Median Total Body Surface Area (m²) for Humans by Age

Age (Years)	Males	Females
3–5	0.728	0.711
6–8	0.931	0.919
9–11	1.16	1.16
12–14	1.49	1.48
15–17	1.75	1.60
Adult	2.09	1.84

Sources: Modified from U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989; U. S. Environmental Protection Agency, *Exposure Factors Handbook: 2011 Edition*, National Center for Environmental Assessment, Washington, DC, EPA/ 600/R-09/052F, 2011.

TABLE 22.47
Total Body Surface Area (m²) For Humans by Height and Weight

Body Weight (kg)	Height (cm)																		260
	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	
5	.18	.20	.23	.26	.29	.33	.37	.42	.48	.55	.62								
10		.35	.36	.38	.41	.44	.48	.52	.57	.64	.69	.76							
15				.54	.57	.57	.60	.63	.67	.72	.77	.83	.89						
20						.68	.72	.76	.80	.85	.91	.97	1.03						
25							.80	.84	.88	.93	.98	1.03	1.09	1.15					
30								.92	.96	1.01	1.05	1.10	1.16	1.22	1.28				
35									1.04	1.08	1.12	1.17	1.23	1.29	1.35	1.42			
40									1.11	1.15	1.20	1.25	1.30	1.36	1.42	1.48	1.55		
45										1.23	1.27	1.32	1.37	1.43	1.48	1.54	1.61		
50										1.30	1.34	1.39	1.44	1.49	1.54	1.60	1.67	1.74	
55										1.37	1.42	1.46	1.50	1.55	1.61	1.67	1.73	1.80	
60										1.44	1.48	1.52	1.57	1.62	1.67	1.73	1.79	1.85	1.92
65											1.54	1.58	1.63	1.68	1.73	1.79	1.85	1.91	1.97
70											1.61	1.65	1.70	1.75	1.80	1.85	1.91	1.96	2.02
75											1.68	1.72	1.76	1.81	1.86	1.91	1.96	2.02	2.08
80											1.74	1.78	1.82	1.86	1.91	1.96	2.02	2.07	2.13
85											1.81	1.84	1.88	1.92	1.97	2.02	2.07	2.13	2.18
90											1.87	1.90	1.94	1.98	2.03	2.08	2.13	2.18	2.24
95												1.97	2.01	2.05	2.09	2.14	2.18	2.24	2.30
100												2.03	2.07	2.12	2.16	2.20	2.24	2.30	2.36
105												2.10	2.14	2.18	2.22	2.26	2.31	2.35	2.41
110												2.17	2.21	2.24	2.28	2.32	2.36	2.41	2.47
115												2.23	2.27	2.30	2.33	2.38	2.42	2.47	2.53
120													2.33	2.36	2.39	2.43	2.48	2.53	2.58
125													2.39	2.42	2.45	2.49	2.53	2.58	2.63
130													2.44	2.47	2.51	2.54	2.59	2.63	2.69
135													2.50	2.53	2.56	2.60	2.64	2.69	2.75
140													2.55	2.58	2.62	2.66	2.70	2.74	2.81
145													2.61	2.63	2.67	2.71	2.75	2.80	2.87
150													2.66	2.69	2.73	2.77	2.81	2.86	2.92
155													2.72	2.74	2.78	2.83	2.87	2.92	2.97
160													2.77	2.80	2.83	2.88	2.92	2.97	3.03
165														2.86	2.89	2.93	2.97	3.02	3.08
170														2.91	2.94	2.98	3.03	3.07	
175														2.96	2.99	3.03	3.08		
180														3.01	3.04	3.08			
185														3.06	3.09				

Source: From Spector, W.S. Ed., *Handbook of Biological Data*, W.B. Saunders, Philadelphia, PA, p. 175, 1956. With permission.

TABLE 22.48
Percentage of Total Body Surface Area by Part

Age (Years)	Head	Trunk	Arms	Hands	Legs	Feet
<1	18.2	35.7	13.7	5.3	20.6	6.5
1 to <2	16.5	35.5	13.0	5.7	23.1	6.3
2 to <3	8.4	41.0	14.4	4.7	25.3	6.3
3 to <6	8.0	41.2	14.0	4.9	25.7	6.4
6 to <11	6.1	39.6	14.0	4.7	28.8	6.8
11 to <16	4.6	39.6	14.3	4.5	30.4	6.6
16 to <21	4.1	41.2	14.6	4.5	29.5	6.1
Adult (male)	6.6	40.1	15.2	5.2	33.1	6.7
Adult (female)	6.2	35.4	12.8	4.8	32.3	6.6

Sources: Modified from U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989; U.S. Environmental Protection Agency, *Exposure Factors Handbook: 2011 Edition*, National Center for Environmental Assessment, Washington, DC, EPA/ 600/R-09/052F, 2011.

TABLE 22.49
Activity Pattern Data Aggregated for Three Microenvironments by Activity Level

Microenvironment	Activity Level	Average Hours in Each Microenvironment at Each Activity Level
Indoors	Resting	9.82
	Light	9.82
	Moderate	0.71
	Heavy	0.098
	Total	20.4
Outdoors	Resting	0.505
	Light	0.505
	Moderate	0.65
	Heavy	0.12
	Total	1.77
In transportation vehicle	Resting	0.86
	Light	0.86
	Moderate	0.05
	Heavy	0.0012
	Total	1.77

Source: U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989.

TABLE 22.50
Summary of Human Inhalation Rates for Men, Women, and Children by Activity Level (m³/h)^a

	Resting ^b	Light ^c	Moderate ^d	Heavy ^e
Adult male	0.7	0.8	2.5	4.8
Adult female	0.3	0.5	1.6	2.9
Average adult ^f	0.5	0.6	2.1	3.9
Child, age 6	0.4	0.8	2.0	2.4
Child, age 10	0.4	1.0	3.2	4.2

Source: From U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989.

^a Values of inhalation rates for males, females, and children presented in this table represent the mean of values reported for each activity level in USEPA (1985).⁶⁰

^b Includes watching television, reading, and sleeping.

^c Includes most domestic work, attending to personal needs and care, hobbies, and conducting minor indoor repairs and home improvements.

^d Includes heavy indoor cleanup, performance of major indoor repairs and alterations, and climbing stairs.

^e Includes vigorous physical exercise and climbing stairs carrying a load.

^f Derived by taking the mean of the adult male and adult female values for each activity level.

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING (FIGURE 22.32; TABLES 22.51 AND 22.52)

This section provides standard values for rodent and human physiological and biochemical parameters used in PBPK modeling. With more emphasis being placed on internal (tissue) dose for quantitating exposure between species, PBPK modeling has found increased use in the risk assessment process.

Figure 22.32 shows a typical PBPK model consisting of a series of compartments representing organ and tissue groups with realistic blood flows. Each tissue group is described mathematically by a series of differential equations, which express the rate of change of a chemical of concern in each compartment. The rate of exchange between compartments is based on species-specific physiological parameters. Unlike data-based pharmacokinetic models, PBPK models are limited within the constraints placed on the model by the unique physiological values for each species modeled. Because PBPK models use species-specific values, interspecies extrapolations can be performed, whereas data-based pharmacokinetic models are limited to intraspecies extrapolation. Herein lies the value of PBPK models in risk assessment.

No one PBPK model can represent the kinetics of all chemicals. The number of compartments and their interrelationships will vary depending on the nature of the chemical being modeled. More specific physiological values than those presented in this section can be used to adjust for variations in strain, sex, age, body weight, etc., of the species being modeled.

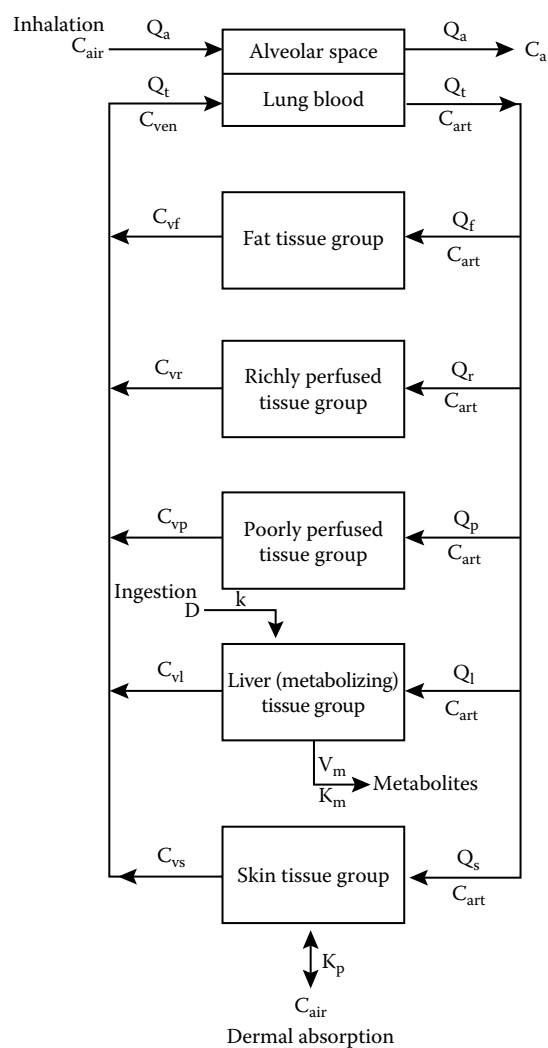


FIGURE 22.32 Diagram of typical PBPK model. C_{art} = concentration in arterial blood (mg/liter); C_{ven} = concentration in venous blood (mg/liter); C_{vf} , C_{vr} , C_{vp} , C_{vl} , C_{vs} = venous concentrations in tissue groups corresponding, respectively, to fat, richly perfused, poorly perfused, liver, and skin tissue groups; C_{air} = concentration in inhaled air (mg/L); C_a = concentration in alveolar air (mg/L); D = gavage dose (mg); and k = gut absorption time constant (min^{-1}). Other abbreviations with reference values can be found in Table 22.51. (From Chen, C.W. and Hoang, K.-C., Incorporating biological information into the assessment of cancer risk to humans under various exposure conditions and issues related to high background tumor incidence rates, Chapter 21, in *Health Risk Assessment: Dermal and Inhalation Exposure and Absorption of Toxicants*, Wang, R.G.M., Knaak, J.B., and Maibach, H.I. Eds., CRC Press, Boca Raton, FL, 1993. With permission.)

TABLE 22.51
Reference Physiological and Biochemical Values Used in PBPK Models

Parameter	Abbreviation	Rats	Mice		Human
			Males	Females	
Body weight (kg)		0.35	0.035	0.025	70
Alveolar ventilation rate (L/min)	Qa	0.083	0.035	0.028	7.5
Blood flow rate (L/min)					
Cardiac output	Qt	0.104	0.023	0.019	6.2
To fat tissue group	Qf	0.0092	0.002	0.0017	0.31
To richly perfused tissue group	Qr	0.0434	0.0012	0.0097	2.76
To poorly perfused tissue group	Qp	0.0074	0.0035	0.00195	1.26
To liver tissue group	Ql	0.0389	0.0058	0.0048	1.55
To skin tissue group	Qs	0.0052	0.00115	0.00095	0.31
Tissue volume (L)					
Of fat tissue group	Vf	0.0315	0.0038	0.0027	14
Of richly perfused tissue group	Vr	0.015	0.0021	0.0015	3.5
Of poorly perfused tissue group	Vp	0.220	0.0273	0.0195	36.4
Of liver tissue group	Vl	0.0140	0.0017	0.0012	1.72
Of skin tissue group	Vs	0.035	0.00035	0.00025	7
Partition coefficient					
Blood/air	N	18.9		16.9	10.3
Skin/air	Ps/a	—		—	505.4
Fat tissue/blood	Pf	108.994		121.893	108.994
Richly perfused tissue/blood	Pr	3.179		4.159	3.719
Poorly perfused tissue/blood	Pp	1.058		1.183	3.72
Liver/blood	Pl	3.719		4.159	3.72
Skin/blood	Ps	—	—		505.4
Metabolic constants					
Maximum velocity of metabolism (mg/min)	Vm	0.00586	0.0039	0.003	0.703
Michaelis constant (mg/L)	Km	2.9378	1.472	1.472	32.043
Absorption coefficients					
Skin permeability (cm/h)	Kp	0.668	—	—	0.17

Source: Modified from Chen, C.W. and Hoang, K.-C., Incorporating biological information into the assessment of cancer risk to humans under various exposure conditions and issues related to high background tumor incidence rates, Chapter 21, in *Health Risk Assessment: Dermal and Inhalation Exposure and Absorption of Toxicants*, Wang, R.G.M., Knaak, J.B., and Maibach, H.I. Eds., CRC Press, Boca Raton, FL, 1993. With permission.

TABLE 22.52
EPA-Recommended Reference Physiological Values for Use in PBPK Modeling

Parameter	Mouse	Rat	Human
Body weight (kg)	0.025	0.25	70.0
Tissue volumes (L)			
Liver	0.0014 (0.055) ^a	0.01 (0.04)	1.82 (0.026)
Fat	0.0025 (0.10)	0.0175 (0.07)	13.3 (0.19)
VRG ^b	0.0013 (0.05)	0.0125 (0.05)	3.5 (0.05)
MG ^c	0.0175 (0.70)	0.1875 (0.75)	43.4 (0.62)
Cardiac output (L/min)	0.017	0.083	6.2
Tissue perfusion (L/min)			
Liver	0.0043 (0.25) ^d	0.0208 (0.25)	1.61 (0.26)
Fat	0.0015 (0.09)	0.0075 (0.09)	0.31 (0.05)
VRG	0.0087 (0.51)	0.0423 (0.51)	2.73 (0.44)
MG	0.0026 (0.15)	0.0125 (0.15)	1.55 (0.25)
Minute volume (L/min)	0.037	0.174	7.5
Alveolar ventilation (L/min)	0.025	0.117	5.0

Source: Modified from U.S. Environmental Protection Agency, *Reference Physiological Parameters in Pharmacokinetic Modeling*, Arms, A.D. and Travis, C.C. Eds., Office of Risk Analysis, EPA No., 600/6-88/004, 1988.

^a Values in parentheses are tissue volumes as fractions of body weight.

^b Vessel-rich group (brain, heart, kidney, viscera).

^c Muscle group (muscle and skin).

^d Values in parentheses are tissue perfusion as fractions of cardiac output.

RISK ASSESSMENT CALCULATIONS

Contained in Table 22.53 are equations for calculations commonly performed in risk assessments. Equations 1 through 4 are for various dose/exposure conversions. Lifetime average daily dose (LADD) for a variety of exposure scenarios can be calculated using Equations 6 through 11. These equations can also be used to calculate maximum daily dose (MDD), sometimes referred to as average daily dose (ADD), by eliminating the lifetime (LT) factor from the denominator and adjusting the numerator for a single day of exposure by removing the exposure duration (ED) factor.

The numerator in Equations 6 through 11 contains absorption (bioavailability) factors to adjust for the fraction of substance actually absorbed (internal dose). Absorption factors may not be found in similar equations from other sources because in the absence of specific data, the value for absorption defaults to one. Additional exposure-modifying factors are used occasionally in the numerator. For example, a meteorological factor for adjusting the exposure for the number of days of precipitation could be used where such an event would limit exposure to dust from contaminated soil. The more data of this type available to express actual exposure, the more accurate will be the calculated estimate of exposure and the more realistic the risk assessment based on this information.

TABLE 22.53

Risk Assessment Calculations

1. Human Equivalent Dose (HED)

$$\text{HED} = (\text{Animal dose}) \times \left[\frac{\text{Animal Body weight}}{\text{Human Body weight}} \right]^{1/3}$$

where

Dose = mg/kg; Body weight = kg

2. ppm-mg/m³ Conversion

$$\text{PPM} = \frac{(\text{mg/m}^3) \times (R)}{(MW)}$$

where

ppm, exposure concentration as ppm; mg/m³, exposure concentration as mg/m³; R, universal gas constant (24.5 at 25°C and 760 mmHg); MW, molecular weight.

3. Airborne Concentration to Equivalent Oral Dose

$$\text{EOD} = \frac{(C) \times (EL) \times (MV) \times (AF) \times (10^{-6})}{(BW)}$$

where

EOD, equivalent oral dose (mg/kg); C, concentration of substance in air (mg/m³); EL, exposure length (min); MV, minute volume, species specific (mL/min); AF, absorption factor (fraction of inhaled substance absorbed), default = 1; 10⁻⁶, conversion factor m³ ↔ mL; BW, body weight (kg)

4. Oral Dose to Equivalent Airborne Concentration*

$$\text{EAC} = \frac{(OD) \times (BW)}{(MV) \times (AF) \times (EL) \times (10^{-6})}$$

where

EAC, equivalent airborne concentration (mg/m³); OD, oral dose (mg/kg); BW, body weight (kg); MV, minute volume, species specific (mL/min); AF, absorption factor, fraction of inhaled substance absorbed; (default = 1) EL, exposure length (min); 10⁻⁶, Conversion factor m³ ↔ mL

* Caution should be exercised when using Equations 3 and 4. These give crude approximations in that the time period will be set and protracted for inhalation and may be either bolus for gavage studies or averaged over the entire day for feeding and drinking water studies. They assume that there will be no chemical reactivity associated with oral administration and no portal entry effects and that the target organ effects will be the same regardless of the route of administration

5. Lifetime Exposure (h)

$$\text{Lifetime exposure} = \left(\frac{\text{hours exposed}}{\text{per day}} \right) \times \left(\frac{\text{days exposed}}{\text{per week}} \right) \times \left(\frac{\text{weeks exposed}}{\text{per year}} \right) \times (\text{years exposed})$$

(continued)

TABLE 22.53 (continued)
Risk Assessment Calculations

6. *Exposure from Ingestion of Contaminated Water*

$$\text{LADD} = \frac{(C) \times (CR) \times (ED) \times (AF)}{(BW) \times (TL)}$$

where

LADD, lifetime average daily dose (mg/kg/day); C, concentration of contaminant in water (mg/L); CR, water consumption rate (L/day); ED, exposure duration (days); AF, absorption factor (fraction of ingested contaminant absorbed) default = 1 (dimensionless); BW, body weight (kg); TL, typical lifetime (days)

7. *Exposure from Dermal Contact with Contaminated Water*

$$\text{LADD} = \frac{(C) \times (SA) \times (EL) \times (AR) \times (ED) \times (SV) \times (10^{-9})}{(BW) \times (TL)}$$

where

LADD, lifetime average daily dose (mg/kg/day); C, concentration of contaminant in water (mg/L); SA, surface area of exposed skin (cm²); EL, exposure length (min/day); AR, absorption rate (μg/cm²/min); SV, specific volume of water (1 L/kg); ED, exposure duration (days); 10⁻⁹, conversion factor (kg ↔ μg); BW, body weight (kg); TL, typical lifetime (days)

8. *Exposure from Ingestion of Contamination Soil*

$$\text{LADD} = \frac{(C) \times (CR) \times (ED) \times (AF) \times (FC) \times (10^{-6})}{(BW) \times (TL)}$$

where

LADD, lifetime average daily dose (mg/kg/day); C, concentration of contaminant in soil (mg/kg); CR, soil consumption rate (mg/day); ED, exposure duration (days); AF, absorption factor (fraction of ingested contaminant absorbed) default 10, (dimensionless); FC, fraction of total soil from contaminated source; 10⁻⁶, conversion factor kg ↔ mg; BW, body weight (kg); TL, typical lifetime (days)

9. *Exposure from Dermal Contact with Contaminated Soil*

$$\text{LADD} = \frac{(C) \times (SA) \times (BF) \times (FC) \times (SDF) \times (ED) \times (10^{-6})}{(BW) \times (TL)}$$

where

LADD (mg/kg/day); C, concentration of contaminant in soil (mg/kg); SA, surface area of exposed skin (cm²); BF, bioavailability factor (percent absorbed/day); FC, fraction of total soil from contaminated source; SDF, soil deposition factor; amount deposited per unit area of skin (mg/cm²/day); ED, exposure duration (days); BW, body weight (kg); TL, typical lifetime (days)

10. *Exposure from Inhalation of Contaminated Particles in Air*

$$\text{LADD} = \frac{(C) \times (PC) \times (IR) \times (RF) \times (EL) \times (AF) \times (ED) \times (10^{-6})}{(BW) \times (TL)}$$

where

LADD, lifetime average daily dose (mg/kg/day); C, concentration of contaminant on particulate (mg/kg); PC, particulate concentration in air (mg/m³); IR, inhalation rate (m³/h); RF, respirable fraction of particulates; EL, exposure length (h/day); AF, absorption factor (fraction of inhaled contaminant absorbed) default = 1; ED, exposure duration (days); 10⁻⁶, conversion factor kg ↔ mg; BW, body weight (kg); TL, typical lifetime (days)

11. *Exposure from Inhalation of Vapors*

$$\text{LADD} = \frac{(C) \times (IR) \times (EL) \times (AF) \times (ED)}{(BW) \times (TL)}$$

where

LADD, lifetime average daily dose (mg/kg/day); C, concentration of contaminant in air (mg/m³); IR, inhalation rate (m³/h); EL, exposure length (h/day); AF, absorption factor (fraction of inhaled contaminant absorbed) default = 1; ED, exposure duration (days); BW, body weight (kg); TL, typical lifetime (days)

TABLE 22.53 (continued)
Risk Assessment Calculations

12. *Calculation of an RfD*

$$\text{RfD} = \frac{(\text{NOAEL})}{(\text{UFs}) \times (\text{MF})}$$

where

RfD (mg/kg/day); UF_s, uncertainty factors generally multiples of 10 (although 3 or 1 are occasionally used depending on the strength and quality of the data). The following UF_s are usually used:

UF

10 accounts for variation in the general population. Intended to protect sensitive subpopulations

10 used when extrapolating from animals to humans. Intended to account for interspecies variability between humans and animals

10 used when a NOAEL is derived from a subchronic rather than a chronic study in calculating a chronic RfD

10 applied when a LOAEL is used instead of a NOAEL. Intended to account for the uncertainty in extrapolating from LOAELs to NOAELs

10 used to account for uncertainty associated with extrapolation when the database is incomplete

MF; multiple of 1–10; intended to reflect a professional qualitative assessment of the uncertainty in the critical study from which the NOAEL is derived as well as the overall quality of the database. Accounts for the uncertainty not addressed by the UF_s. The use of the MF was discontinued in 2004 as it was felt that the uncertainties addressed by this factor are covered by the UF_s

13. *Estimating an LD₅₀ of a Mixture*

$$\frac{1}{\text{Predicted LD}_{50}} = \frac{P_a}{\text{LD}_{50} \text{ of Component a}} = \frac{P_b}{\text{LD}_{50} \text{ of Component b}} + \dots + \frac{P_n}{\text{LD}_{50} \text{ of Component n}}$$

where

P, fraction of components in the mixture

14. *Estimation of Maximal Attainable Air Concentration of a Chemical*

$$\text{MAAC} = \frac{(\text{vp}) \times (\text{mw}) \times (10^6)}{(760) \times (R)}$$

where

MAAC, maximal attainable air concentration (mg/m³); vp, vapor pressure of the chemical (mmHg) at 25°C; mw, molecular weight in grams; 760, atmospheric pressure at 25°C; R, 24.5 (universal gas constant at 25°C and 760 mmHg)

Note: In the absence of analytical measurements, this equation can give a worst-case estimate of the theoretically achievable air concentration of a volatile chemical at equilibrium in any size room with no ventilation and an infinite source of chemical. Actual air concentrations could be lower depending on physical properties of the chemical, the amount of chemical being used, room ventilation, and other handling practices but not exceed the MAAC

15. *Haber's Rule*

$$C \times t = k$$

where

C, exposure concentration; t, time; and k, a constant

Note: Haber's rule has been historically used to relate exposure concentration and duration to a toxic effect. Basically, this concept states that exposure concentration and exposure duration (ED) may be reciprocally adjusted to maintain a cumulative exposure constant (k) and that this cumulative exposure constant will always reflect a specific toxic response. In general terms, it states that the shorter the time of exposure, the higher the concentration that will be needed to achieve the same toxic effect as occurs with a longer period of exposure at lower concentrations. The inverse relationship of concentration and time may be valid when the toxic response to a chemical is equally dependent upon the concentration and the ED. However, work by ten Berge et al. (1986) with acutely toxic chemicals revealed chemical-specific relationships between exposure concentration and exposure time that were often exponential rather than linear. This relationship can be expressed by the Equation $C^n \times t = k$, where n represents a chemical specific, and even toxic end point specific, exponent. The relationship described by this equation is basically the form of a linear regression analysis of the log–log transformation of a plot of C vs. t. ten Berge et al. examined the airborne concentration (C) and short-term exposure time (t) relationship relative to lethal responses for approximately 20 chemicals and found that the empirically derived value of n ranged from 0.8 to 3.5 among this group of chemicals. Hence, these workers showed that the value of the exponent (n) in the equation $C^n \times t = k$ quantitatively defines the relationship between exposure concentration and ED for a given chemical and for a specific toxic or health effect end point. Haber's rule is the special case where $n = 1$. As the value of n increases, the plot of concentration vs. time yields a progressive decrease in the slope of the curve. In short, the best expression for extrapolation over several time points is $C^n \times t = k$, where the value for n is derived from existing data (Standing Operating Procedures of the National Advisory Committee on Acute Exposure Guideline Levels for Hazardous Substances, 2001⁷⁴)

(continued)

TABLE 22.53 (continued)
Risk Assessment Calculations

16. *Time-Weighted Average (TWA) for an 8 h Workday*

$$\text{TWA} = \frac{C_1T_1 + C_2T_2 + \dots C_nT_n}{8}$$

where

C_n , concentration measured during a period of time (<8 h); T_n , duration of the period of exposure in hours at concentration C_n ($\Sigma T = 8$)

17. *Risk for Noncarcinogens (Hazard Index)*

$$\text{Risk} = \frac{\text{MDD}}{\text{ADI}}$$

If: Risk > 1, a potential risk exists, which may be significant.

Risk < 1, risk is insignificant.

where

MDD, maximum daily dose; ADI, acceptable daily intake

18. *Lifetime Risk for Carcinogens*

$$\text{Risk} = (\text{LADD}) \times (\text{SF})$$

If: Risk = 10^{-6} , risk is insignificant; 10^{-6} – 10^{-4} , possible risk; 10^{-4} , risk may be significant.

where

LADD, lifetime average daily dose (mg/kg/day); SF, slope factor or cancer potency factor (mg/kg/day)⁻¹ (chemical and route specific)

19. *Total Risk from a Single Contaminant via Multiple Exposure Pathways*

$$\text{Total} = \Sigma \text{ risks from all exposure pathways}$$

Example: Total risk (from a contaminant in water) = (risk from ingestion) + (risk from showering) + (risk from swimming)

20. *Total Risk from Multiple Contaminants via a Single Exposure Pathway*

$$\text{Total risk} = \Sigma \text{ risks from all contaminants in the media}$$

Example:

Total risk from contaminants A, B, and C in water = total risk from contaminant A + total risk from contaminant B + total risk from contaminant C

For calculations 19 and 20: total risk < 1 is insignificant; total risk > 1 may be significant. Both of these methods are extremely conservative and can greatly overestimate risk.

Sources: From Paustenbach, D.J. and Leung, H.-W., Techniques for assessing the health risks of dermal contact with chemicals in the environment, Chapter 23, in *Health Risk Assessment: Dermal and Inhalation Exposure and Absorption of Toxicants*, Wang, R.G.M., Knaak, J.B., and Maibach, H.I. Eds., CRC Press, Boca Raton, FL, 1993; Environ Corporation, *Risk Assessment Guidance Manual*, Allied Signal Inc., Morristown, NJ, 1990; U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J.J., Lisi, K., Friebele, E., and Dixon, D.A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989; U.S. Environmental Protection Agency, *Risk Assessment Guidance for Superfund, Vol. 1: Human Health Evaluation Manual*, Office of Emergency and Remedial Response, EPA No. 540/1-89/002, 1989; Lynch, J.R., Measurement of worker exposure, Chapter 6, in *Patty's Industrial Hygiene and Toxicology, Vol. III. Theory and Rationale of Industrial Hygiene Practice*, Cralley, L.V. and Cralley, L.J. Eds., John Wiley & Sons, New York, 1979.

REFERENCES

1. U.S. Environmental Protection Agency, *General Quantitative Risk Assessment Guidance for Non-Cancer Health Effects*, ECAP-CIN-538M, 1989, cited in Hooper et al.³
2. National Research Council, *Risk Assessment in the Federal Government*, National Academy Press, Washington, DC, 1983.
3. Hooper, L. D., Oehme, F. W., and Krieger, G. R., Risk assessment for toxic hazards, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R. Eds., Williams & Wilkins, Baltimore, MD, 1992, Chapter 7, pp. 65–76.
4. Hallenbeck, W. H. and Cunningham K. M., Qualitative evaluation of human and animal studies, in *Quantitative Risk Assessment for Environmental and Occupational Health*, Lewis Publishers, Chelsea, MI, 1986, Chapter 3.
5. Zbinden, G., *Progress in Toxicology*, Vol. 1, Springer-Verlag, New York, 1973.
6. Ecobichon, D. J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 2.
7. Ballantyne, B. and Sullivan, J. B., Basic principles of toxicology, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R. Eds., Williams & Wilkins, Baltimore, MD, 1992, Chapter 2, pp. 9–23.
8. Paul, M., Clinical evaluation and management, in *Occupational and Environmental Reproductive Hazards: A Guide for Clinicians*, Williams & Wilkins, Baltimore, MD, 1993, Chapter 10.
9. Chow, A. W. and Jewesson, P. J., Pharmacokinetics and safety of antimicrobial agents during pregnancy, *Rev. Infect. Dis.*, 7, 288, 1985.

10. McGuigan, M. A., Teratogenesis and reproductive toxicology, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R. Eds., Williams & Wilkins, Baltimore, MD, 1992, Chapter 16, pp. 179–189.
11. Lowe, J. A. et al., *Health Effects of Municipal Waste Incineration*, CRC Press, Boca Raton, FL, 1990.
12. U.S. Environmental Protection Agency, *Techniques for the Assessment of the Carcinogenic Risk to the U.S. Population due to Exposure from Selected Volatile Organic Compounds from Drinking Water via the Ingestion, Inhalation and Dermal Routes*, Cothorn, C. R., Coniglio, W. A., and Marcus, W. L. Eds., Office of Drinking Water, NTIS, PB 84-213941, 1984.
13. U.S. Environmental Protection Agency, *Reported in Pesticide and Toxic Chemical News*, October 19, p. 34, 1988.
14. Morgan, M. G., Risk analysis and management, *Scientific Am.*, 269, 32, 1993.
15. Lowrance, W. M., *Of Acceptable Risk: Science and the Determination of Safety*, William Kaufmann, Los Altos, CA, 1976, Chapter 3.
16. Ballantyne, B., Exposure-dose-response relationships, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R. Eds., Williams & Wilkins, Baltimore, MD, 1993, Chapter 3, pp. 24–30.
17. Klaassen, C. D. and Eaton, D. L., Principles of toxicology, in *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th edn., Doull, J., Klaassen, C. D., and Anders, M. O. Eds., Pergamon Press, New York, 1991, Chapter 2, pp. 12–49.
18. Ecobichon, D. J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 7.
19. Crone, H. D., *Chemicals and Society: A Guide to the New Chemical Age*, Cambridge University Press, Cambridge, U.K., 1986, Chapter 4.
20. Munro, I. C. and Krewski, D. R., Risk assessment and regulatory decision making, *Food Cosmet. Toxicol.*, 19, 549, 1981.
21. Van Ryzin, J., Quantitative risk assessment, *J. Occup. Med.*, 22, 321, 1980.
22. Van Ryzin, J. and Rai, K., The use of quantal response data to make predictions, in *The Scientific Basis of Toxicity Assessment*, Witschi, H. R. Ed., Elsevier/North-Holland, New York, 1980, p. 273.
23. Selevan, S. G., Epidemiology, in *Occupational and Environmental Reproductive Hazards: A Guide for Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993, Chapter 9, pp. 100–110.
24. Piantadosi, S., Epidemiology and principles of surveillance regarding toxic hazards in the environment, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R. Eds., Williams & Wilkins, Baltimore, MD, 1992, Chapter 6, pp. 61–64.
25. U. S. Environmental Protection Agency, *Interim Methods for the Development of Inhalation Reference Doses*, Blackburn, K., Dourson, M., Erdreich, L., Jarabek, A. M., and Overton, J. Jr. Eds., Environmental Criteria and Assessment Offices, EPA1600/8-88/066F, 1989.
26. Gamble, J. F. and Battigelli, M. C., Epidemiology, in *Patty's Industrial Hygiene and Toxicology*, 3rd revised edn., Vol. I, Clayton, G. D. and Clayton, F. E. Eds., John Wiley & Sons, New York, 1978, Chapter 5, pp. 113–134.
27. Piantadosi, S. and Sullivan, J. B., Chemical and environmental carcinogenesis, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B. and Krieger, G. R. Eds., Williams & Wilkins, Baltimore, MD, 1992, Chapter 8, pp. 77–84.
28. IARC Working Group on the Evaluation of Carcinogenic Risk, Overall evaluations of carcinogenicity: An updating of IARC monographs, in *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 1–42 (Supplement 7), IARC Working Group on the Evaluation of Carcinogenic Risk, Lyon, France, 1987.
29. Meijers, J. M. M., Swaen, G. M. H., Schreiber, G. H., and Sturmans, F., Occupational epidemiological studies in risk assessment and their relation to animal experimental data, *Regul. Toxicol. Pharmacol.*, 16, 215, 1992.
30. Doll, B. and Peto, R., The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today, *J. Natl. Cancer Inst.*, 68, 1191, 1981.
31. Higginson, J. and Muir, C. S., Environmental carcinogenesis: Misconceptions and limitations to cancer control, *J. Natl. Cancer Inst.*, 63, 1291, 1979.
32. Wynder, E. L. and Gori, G. B., Contribution of the environment to cancer incidence: An epidemiologic exercise, *J. Natl. Cancer Inst.*, 58, 825, 1977.
33. Weisburger, E. K., Industrial and environmental cancer risks, in *Dangerous Properties of Industrial Materials*, 6th edn., Sax, N. I. Ed., Van Nostrand Reinhold, New York, 1984, Section 3.
34. Ries, L. A. G., Hankey, B. F., and Edwards, B. K. Eds., *Cancer Statistics Review, 1973–1987*, National Cancer Institute, Bethesda, MD, 1990.
35. Huff, J., Cirvello, J., Haseman, J., and Bucher, J., Chemicals associated with site-specific neoplasia in 1394 long term carcinogenesis experiments in laboratory rodents, *Environ. Health Perspect.*, 93, 247, 1991.
36. American Cancer Society, *Cancer Facts and Figures*, American Cancer Society, Okaland, CA, 2000.
37. American Cancer Society, *Cancer Facts and Figures*, American Cancer Society, Okaland, CA, 2012.
38. U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program, *Annual Report on Carcinogens*, 12th Edn., 2011.
39. Warburton, I., Measurement of reproductive effects in human populations: Selected outcomes for study, in *Symposium on Criteria for Assessment of Health Effects at Chemical Disposal Sites*, Rockefeller University, New York, 1981.
40. Kline, J., Stein, Z., and Susser, M., *Conception to Birth: Epidemiology of Prenatal Development*, Oxford University Press, New York, 1989.
41. Shepard, T. H., Fantel, A. G., and Mirkes, P. E., Developmental toxicology: Prenatal period, in *Occupational and Environmental Reproductive Hazards: A Guide to Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993, Chapter 4, pp. 37–50.
42. Persaud, T. V. N., Teratogenic mechanisms, in *Advances in the Study of Birth Defects*, Vol. 1, University Park Press, Baltimore, MD, 1979.
43. Rousseaux, C. G. and Blakley, P. M., Fetus, in *Handbook of Toxicologic Pathology*, Haschek, W. M., and Rousseaux, C. G. Eds., Academic Press, San Diego, CA, 1991, Chapter 25.
44. National Foundation/March of Dimes: Report of Panel II. Guidelines for reproductive studies in exposed human populations, in *Guidelines for Studies of Human Populations Exposed to Mutagenic and Reproductive Hazards*, Bloom, A. D. Ed., The Foundation, New York, 1981, p. 37.
45. Manson, J. M. and Wise, L. D., Teratogens, in *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th edn., Amdur, M. O., Doull, J., and Klaassen, C. D. Eds., Pergamon Press, New York, 1991, Chapter 7, pp. 226–254.

46. Moore, K. L., *The Developing Human: Clinically Oriented Embryology*, 5th edn., Saunders, Philadelphia, PA, 1993.
47. Kline, J. and Stein, Z., Very early pregnancy, in *Reproduction Toxicology*, Dixon, R. L. Ed., Raven Press, New York, 1985, p. 259.
48. Wilson, J. G., Teratogenic effects of environmental chemicals, *Fed. Proc.*, 36, 1698, 1977.
49. Ecobichon, D. J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 5.
50. Shepard, T. H., Fantel, A. G., and Fitzsimmon, J., Congenital defect rates among spontaneous abortuses: Twenty years of monitoring, *Teratology*, 39, 325, 1989.
51. Fantel, A. G. and Shepard, T. H., Morphological analysis of spontaneous abortuses, in *Spontaneous and Recurrent Abortion*, Bennett, M. J. and Edmons, D. K. Eds., Blackwell Publications, Oxford, U.K., 1987, p. 8.
52. MMWR, Annual summary 1981, *Morb. Mortal. Wkly. Rep.*, 30(54), 108, 1981.
53. Crone, H. D., *Chemicals and Society: A Guide to the New Chemical Age*, Cambridge University Press, Cambridge, U.K., 1986, Chapter 7.
54. National Research Council, *Improving Risk Communication*, Committee on Risk Perception and Communication, Commission on Behavioral and Social Sciences and Education, Commission on Physical Sciences, Mathematics and Resources, National Academy Press, Washington, DC, 1989.
55. Crouch, E. A. C. and Wilson, R., Inter-risk comparisons, in *Assessment and Management of Chemical Risks*, Rodricks, J. V. and Tardiff, R. G. Eds., American Chemical Society, Washington, DC, 1984, Chapter 7.
56. Paget, G. E. Ed., *Methods in Toxicology*, Blackwell Scientific Publishers, Oxford, U.K., 1970, p. 49.
57. Ecobichon, D. J., *The Basis of Toxicity Testing*, CRC Press, Boca Raton, FL, 1992, Chapter 4.
58. Kleiber, M., *The Fire of Life: An Introduction to Animal Energetics*, John Wiley & Sons, New York, 1961.
59. Mortensen, M. E., Mercury toxicity in children, in *Similarities and Differences between Children and Adults: Implications for Risk Assessment*, Guzelian, P. S., Henry, C. J., and Olin S. S. Eds., ISLI Press, Washington, DC, 1992, p. 204.
60. U.S. Environmental Protection Agency, *Development of Statistical Distributions or Ranges of Standard Factors Used in Exposure Assessments*, Office of Health and Environmental Assessments, EPA No. 600/8-85/010, NTIS, PB85-242667, 1985.
61. U.S. Environmental Protection Agency, *Reference Physiological Parameters in Pharmacokinetic Modeling*, Arms, A. D. and Travis, C. C. Eds., Office of Risk Analysis, EPA No., 600/6-88/004, 1988.
62. Plowchalk, D., Meadows, M. J., and Martinson, D. R., Comparative approach to toxicokinetics, In: *Occupational and Environmental Reproductive Hazards, A Guide for Clinicians*, Paul, M. Ed., Williams & Wilkins, Baltimore, MD, 1993, Chapter 3.
63. U.S. Environmental Protection Agency, *Exposure Factors Handbook*, Konz, J. J., Lisi, K., Friebele, E., and Dixon, D. A. Eds., Office of Health and Environmental Assessments, EPA No. 600/8-89/043, 1989.
64. Spector, W. S. Ed., *Handbook of Biological Data*, W.B. Saunders, Philadelphia, PA, 1956, p. 175.
65. Chen, C. W. and Hoang, K.-C., Incorporating biological information into the assessment of cancer risk to humans under various exposure conditions and issues related to high background tumor incidence rates, in *Health Risk Assessment: Dermal and Inhalation Exposure and Absorption of Toxicants*, Wang, R. G. M., Knaak, J. B., and Maibach, H. I. Eds., CRC Press, Boca Raton, FL, 1993, Chapter 21, pp. 309–329.
66. U.S. Environmental Protection Agency, *Risk Assessment Guidance for Superfund, Vol. 1: Human Health Evaluation Manual*, Office of Emergency and Remedial Response, EPA No. 540/1-89/002, 1989.
67. Paustenbach, D. J. and Leung, H.-W., Techniques for assessing the health risks of dermal contact with chemicals in the environment, in *Health Risk Assessment: Dermal and Inhalation Exposure and Absorption of Toxicants*, Wang, R. G. M., Knaak, J. B., and Maibach, H. I. Eds., CRC Press, Boca Raton, FL, 1993, Chapter 23, pp. 343–385.
68. Lynch, J. R., Measurement of worker exposure, in *Patty's Industrial Hygiene and Toxicology, Vol. III. Theory and Rationale of Industrial Hygiene Practice*, Cralley, L. V. and Cralley, L. J. Eds., John Wiley & Sons, New York, 1979, Chapter 6, pp. 217–255.
69. Environ Corporation, *Risk Assessment Guidance Manual*, Allied Signal Inc., Morristown, NJ, 1990.
70. American Cancer Society, *Global Cancer Facts and Figures*, 2nd edn., American Cancer Society, Atlanta, GA, 2011.
71. The Mountain States Genetic Foundation, *The Genetic Drift Newsletter*, Vol. 12, The Mountain States Genetic Foundation, 1995.
72. Farrer, F., Contraception for teratogenic medications, *SA Pharm. J.*, 10, 28, 2010.
73. U. S. Environmental Protection Agency, *Exposure Factors Handbook: 2011 Edition*, National Center for Environmental Assessment, Washington, DC, EPA/ 600/R-09/052F, 2011.
74. *Standing Operating Procedures of the National Advisory Committee on Acute Exposure Guideline Levels for Hazardous Substances*, National Academy Press, Washington, DC, 2001.
75. *Low-Dose Extrapolation of Cancer Risks: Issues and Perspectives*, International Life Sciences Institute, Washington, DC, 1995, p. 188.
76. Gargas, M. L., Finley, B. L., Pustenbach, D. J., and Long, T. F., Environmental health risk assessment: Theory and practice, in *General and Applied Toxicology*, Ballantyne, B., Marrs, T. C., and Syversen, T. Eds., Grove's Dictionary, New York, 1999, Chapter 82, pp. 1749–1809.
77. Morselli, P. L., Clinical pharmacology of the prenatal period and early infancy, *Clin. Pharmacokinet.*, 17(Suppl 1), 13, 1989.
78. Ritschel, W. A. and Kearns, G. L., *Handbook of Basic Pharmacokinetics*, 5th edn., American Pharmaceutical Association, Washington, DC, 1999.
79. Leeder, J. S. and Kearns, G. L., Pharmacogenetics in pediatrics: Implications for practice, *Pediatr. Clin. North Am.*, 44, 55, 1997.
80. U. S. Environmental Protection Agency, *Exposure Factors Handbook*, Wood, P., Phillips, L., Adenuga, A., Koontz, M., Rector, H., Wilkes, C., and Wilson, M. Eds., National Center for Environmental Assessment, EPA/ 600/p-95/002Fa, 1997.

23 Regulatory Toxicology

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INTRODUCTION

This chapter presents a general overview of regulatory toxicology primarily related to the United States and the European Union (EU). More detailed information on regulations and guidelines related to specific types of products and areas of toxicology can be found in other chapters in this book as outlined in Table 23.1. Much of the information presented in this chapter was consolidated from several chapters in the second edition of the *Handbook of Toxicology*

authored by the following individuals: Michael J. Derelanko (US Overview), Henry C. Fogle (US EPA/Chemicals [TSCA]), Jane E. Harris (US EPA/Pesticides [FIFRA]), Steven J. Hermansky (Medical Devices), and Dennis J. Naas (Consumer Products). Regulatory laws, regulations, and guidelines frequently change. Therefore, it is important for the reader to verify that any regulatory information provided in this book is still applicable for his/her current and future situation and needs.

TABLE 23.1
Regulatory References in Other Chapters

	Chapter	Section	Topic
1	Laboratory Animal Management	3	Regulations and Guidelines
2	General Toxicology	3	Regulatory Guidelines
3	Dermal Toxicology	2	Comparison of Regulatory Guidelines
4	Ocular Toxicology	3	Regulatory Guidelines
7	Neurotoxicology	2	Regulatory Guidelines
8	Immunotoxicology	2	Regulatory Agencies/Guidelines
10	Reproductive/Developmental Toxicology	1/Refs.	Agencies, Guidelines
11	Juvenile Toxicology	2	Regulatory Agencies/Guidelines
12	Endocrine Toxicology	3	National and International Regulations
13	Genetic Toxicology	9	Regulatory Guidelines
19	Chemical Toxicology	2	General Information, OECD Guidelines
20	Pharmaceutical	Several	FDA Guidelines
21	Safety Pharmacology	2	Harmonized Guidelines

OVERVIEW OF REGULATORY TOXICOLOGY IN THE UNITED STATES

Tables 23.2 through 23.4 present an overview of US federal agencies and major legislation regulating toxic substances.¹⁻³ More detailed information is presented

elsewhere in the handbook or can be obtained from the references cited. Other legislation regulating toxic substances exists at the federal, state, and local levels, but a discussion of such legislation is beyond the scope of this book.

TABLE 23.2
Principal US Regulatory Agencies Having Involvement with Toxicology

Agency	Agency Description	General Coverage
Food and Drug Administration (FDA)	A unit of the Department of Health and Human Services	<ul style="list-style-type: none"> • Drugs and foods • Food additives and cosmetics • Medical devices • Biologicals
Environmental Protection Agency (EPA)	Independent agency, not a part of a cabinet department	<ul style="list-style-type: none"> • Pesticides and sterilants • Industrial chemicals • Air pollutants • Industrial waste
Occupational Safety and Health Administration (OSHA)	Unit of the Department of Labor	<ul style="list-style-type: none"> • Occupational exposure
CPSC	Independent commission	<ul style="list-style-type: none"> • Consumer products
USDA	Cabinet department	<ul style="list-style-type: none"> • Veterinary biologicals • Administers the Animal Welfare Act covering the well-being and humane treatment of warm-blooded lab animals. Currently, birds, rats, and mice are excluded. But efforts are underway that may result in their inclusion in the future

TABLE 23.3
Major US Federal Legislation Involving Toxic Substances

Statute	Code of Federal Regulations (CFR) Citation	Description
Clean Air Act (CAA)	40 CFR 50–80	Administered by EPA. Deals with control of hazardous air pollutants. Sets national standards for air quality, sources that produce air pollution, emission of noxious air pollutants, and motor vehicles
Clean Water Act (CWA)	40 CFR 100–140, 400–470	Administered by EPA. Limits water pollution from industrial and municipal sources, provides funding for municipal sewage treatment construction, allows recovery of costs in mitigation of hazardous substance spills, emphasizes the importance of controlling toxic pollutants, and encourages waste treatment experimentation
Comprehensive Environmental Response, Compensation and Liability Act (CERCLA)	40 CFR 300	Administered by EPA. Known as “Superfund.” Requires cleanup of hazardous substances released into air, water, and land; covers both new releases and old dumpsites; establishes reportable quantities (RQs) for certain hazardous substances
Consumer Product Safety Act (CPSA)	16 CFR 1015–1402	Administered by CPSC. Regulates products that pose unreasonable risk of injury or illness to consumers, establishes safety standards, promotes research into causes and prevention of product-related deaths, illness, and injuries
Federal Food, Drug and Cosmetic Act (FFDCA)	21 CFR 1–1300	Administered by FDA. Forbids the marketing of any food containing additives or non additives that render it injurious to health; requires the safety of food additives to be demonstrated; requires premarket approval of all new drugs based on demonstration of safety and efficacy; regulates the testing, marketing, and use of medical devices; regulates the distribution of cosmetics that pose a risk of more than transitory harm if used as intended; and mandates safety testing of color additives.
Federal Hazardous Substances Act (FHSA)	16 CFR 1500–1512	Administered by CPSC. Regulates, primarily through labeling requirements, hazardous substances used by consumers that are toxic, corrosive, combustible, and radioactive or generate pressure. Contains detailed criteria for determining toxicity
Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)	40 CFR 162–180	Administered by EPA. Regulates all pesticides marketed in the United States through registration requirements. Safety and efficacy must be demonstrated such that no unreasonable risk to man and the environment is indicated
Food Quality Protection Act (FQPA)	—	Administered by EPA. Amends FIFRA and FFDCA. Intended to update and resolve inconsistencies in the two major pesticide statutes; regulates pesticides to a reasonable-certainty-of-no-harm safety standard; mandates a single, health-based standard for all pesticides in all foods; provides special protection of infants and children; expedites approval of safer pesticides; creates incentives for the development of effective crop protection tools; requires periodic reevaluation of pesticide registrations and tolerances; and addresses screening for endocrine active substances
Hazardous Materials Transportation Act (HMTA)	49 CFR 106–107, 171–179	Administered by the Department of Transportation (DOT). Regulates hazardous materials shipped by road, air, or rail; specifies packaging, labeling, and shipping requirements
Occupational Safety and Health Act (OSHA)	29 CFR 1910, 1915, 1918, 1926	Administered by the Occupational Safety and Health Act (OSHA). Requires employers to provide safe working conditions, prescribes mandatory occupational safety and health standards including exposure limits for toxic chemicals, requires assessment of chemical hazards and notification of workers of such hazards (requires material safety data sheets). Established the National Institutes of Occupational Safety and Health (NIOSH)
Poison Prevention Packaging Act (PPPA)	16 CFR 1700–1704	Administered by CPSC. Sets standards for packaging of hazardous household products
Resource Conservation and Recovery Act (RCRA)	40 CFR 240–271	Administered by EPA. Regulates the activities of generators, transporters, and those who treat, store, or dispose hazardous wastes; establishes a list of waste substances considered hazardous; establishes a manifest system to track the generation, transportation, and disposal of hazardous wastes. Under RCRA, methods for treating, storing, and disposing of wastes are prescribed; location design and construction of treatment facilities are governed; qualifications for ownership, training, and financial responsibility of treatment facilities are established
Safe Drinking Water Act (SDWA)	40 CFR 140–149	Administered by EPA. Sets standards for drinking water to protect public health. Maximum contaminant levels (MCLs) are prescribed. Includes provisions specifically designed to protect underground sources of drinking water. As of August 1996, amended to address screening of endocrine active substances
Toxic Substances Control Act (TSCA)	40 CFR 700–799	Administered by EPA. Regulates the production, processing, importation, and use of chemical substances that present an unreasonable risk to health or the environment; requires notification of production of a new chemical or significant new use of an existing chemical; testing may be required for hazard assessment for high volume/high exposure chemicals; requires record keeping and reporting requirements of significant health effects

TABLE 23.4
Federal Regulatory Programs Involved with Toxicological Testing

Agency	Authority	Statute	Program
CPSC	Consumer product exposures	FHSA; CPSA; PPPA	Hazard Assessment and Reduction Program and Regulated Products Program
DOI ^a	Drug and management chemical for fisheries	Fish and Wildlife Coordination Act; FIFRA; FFDCA	Chemical-Drug Registration Program, National Biological Survey
	Nontoxic Shot Program	Migratory Bird Treaty Act	Office of Migratory Bird Management, Fish and Wildlife Service
DOT ^b	Exposure to hazardous materials in transport	Federal Hazardous Materials Transportation Law	Research and Special Programs Administration
EPA	Pesticides	FIFRA	Office of Pesticide Programs (OPP)
	Industrial chemicals	TSCA	Office of Pollution Prevention and Toxics
FDA	Biologicals	FFDCA; Public Health Service Act	Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER)—gene therapy, some vaccines, blood products
	Medical devices	FFDCA	Center for Devices and Radiological Health
	Radioactive materials		
	Pharmaceuticals	FFDCA	Center for Drug Evaluation and Research
	Food and color additives, cosmetics	FFDCA	Center for Food Safety and Applied Nutrition
	Veterinary drugs	FFDCA	Center for Veterinary Medicine
OSHA	Worker exposures	OSHA	Directorate of Health Standards Programs
USDA	Genetically engineered plants, microbes, and arthropods	Plant Pest Act	APHIS ^c
	Veterinary biologicals and diagnostics	Virus–Serum–Toxin Act	APHIS ^c
	Nonfood compounds on foods	Federal Meat Inspection Act; Poultry Products Inspection Act	Food Safety Inspection Service

Source: ICCVAM, ntp-server.niehs.nih.gov/htdocs/iccvam/REGUL.html.

^a Department of the Interior.

^b Department of Transportation.

^c Animal and Plant Health Inspection Service. The program has authority, but no routine toxicity testing requirements.

CHEMICALS

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

The US Environmental Protection Agency (US EPA) was created in 1970. It is the federal agency responsible for the administration of environmental protection laws in the United States including the Toxic Substances Control Act (TSCA), US Public Law 94-469. TSCA was created by the US Congress in 1976 to “protect human health and the environment by requiring testing and necessary use restrictions on certain chemical substances and for other purposes.”¹ This law became effective in the United States on January 1, 1977, and is codified in Title 40 of the US Code of Federal Regulations (CFR).

The EPA’s Office of Chemical Safety and Pollution Prevention (OCSPP), formerly called the Office of Prevention, Pesticides, and Toxic Substances (OPPTS), works directly with TSCA and Federal Insecticide,

Fungicide, and Rodenticide Act (FIFRA) implementation. This office “... is responsible for the development of national strategies for the control of toxic substances; criteria for assessing chemical substances; standards for test protocols for chemicals; rules and procedures for industry reporting and regulations for the control of substances deemed to be hazardous to man or the environment; enforcement of standards; and evaluating and assessing the impact of new chemicals and chemicals with new uses to determine the potential risk to health and/or the environment and, if needed, develop appropriate restrictions. The EPA coordinates activities on the assessment and control of toxic substances. Additional activities include control and regulation of pesticides and reduction in their use to assure human safety and protection of environmental quality; establishment of tolerance levels for pesticides that occur in or on food; monitoring of pesticide residue levels in food, humans, and nontarget fish, wildlife and

their environments; investigation of pesticide accidents; and coordination of the Agency pollution prevention program.”⁴

The OCSPP has developed a series of harmonized test guidelines for use in the testing of pesticides and toxic substances and the development of test data for submission to the Agency.⁵ The OCSPP harmonized test guidelines are documents that specify methods that EPA recommends be used to generate data that are submitted to EPA to support the registration of a pesticide under FIFRA (7 USC 136), setting of a tolerance or tolerance exemption for pesticide residues under Section 408 the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 USC 346a), or the decision-making process for an industrial chemical under TSCA (15 USC 2601). The OCSPP harmonized test guidelines are organized in the following series:

- 810—Product Performance Test Guidelines
- 830—Product Properties Test Guidelines
- 835—Fate, Transport and Transformation Test Guidelines
- 840—Spray Drift Test Guidelines
- 850—Ecological Effects Test Guidelines
- 860—Residue Chemistry Test Guidelines
- 870—Health Effects Test Guidelines
- 875—Occupational and Residential Exposure Test Guidelines
- 880—Biochemicals Test Guidelines
- 885—Microbial Pesticide Test Guidelines
- 890—Endocrine Disruptor Screening Program Test Guidelines

OCSPP health effects test guidelines (Series 870) for toxicity testing⁶ are listed in Table 23.5. Note—the name change from “Office of Prevention, Pesticides, and Toxic Substances” and “OPPTS” to “Office of Chemical Safety and Pollution Prevention” and “OCSPP” does not affect the guidelines.

Studies conducted according to these test guidelines may be used for satisfying FIFRA data requirements in 40 CFR Part 158 and Part 161, data call-ins issued pursuant to FIFRA Section 3(c)(2)(B), as needed to satisfy data requirements appropriate for specific pesticide registration applications, or for satisfying data requirements to demonstrate the safety of a tolerance or tolerance exemption under FFDCA Section 408.

Test guidelines used in regulatory actions as bases for test standards under TSCA are typically promulgated in 40 CFR Part 799 or may be written into specific TSCA rules (such as test rules under TSCA Section 4). The test guidelines may also be used as part of voluntary testing. Note that where data will be required under a TSCA rule (such as a test rule under TSCA Section 4), a TSCA-specific version of the applicable guideline may be promulgated as a rule. Examples may be found at 40 CFR Part 799, subparts E and H.

TABLE 23.5

Office of Chemical Safety and Pollution Prevention (OCSPP) Harmonized Test Guidelines

Group A—Acute Toxicity Test Guidelines

- 870.1000—Acute Toxicity Testing—Background (December 2002)
- 870.1100—Acute Oral Toxicity (December 2002)
- 870.1200—Acute Dermal Toxicity (August 1998)
- 870.1300—Acute Inhalation Toxicity (August 1998)
- 870.2400—Acute Eye Irritation (August 1998)
- 870.2500—Acute Dermal Irritation (August 1998)
- 870.2600—Skin Sensitization (March 2003) (PDF)

Group B—Subchronic Toxicity Test Guidelines

- 870.3050—Repeated Dose 28-Day Oral Toxicity Study in Rodents (July 2000)
- 870.3100—90-Day Oral Toxicity in Rodents (August 1998)
- 870.3150—90-Day Oral Toxicity in Nonrodents (August 1998)
- 870.3200—21/28-Day Dermal Toxicity (August 1998)
- 870.3250—90-Day Dermal Toxicity (August 1998)
- 870.3465—90-Day Inhalation Toxicity (August 1998)
- 870.3550—Reproduction/Developmental Toxicity Screening Test (July 2000)
- 870.3650—Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (July 2000)
- 870.3700—Prenatal Developmental Toxicity Study (August 1998)
- 870.3800—Reproduction and Fertility Effects (August 1998)

Group C—Chronic Toxicity Test Guidelines

- 870.4100—Chronic Toxicity (August 1998)
- 870.4200—Carcinogenicity (August 1998)
- 870.4300—Combined Chronic Toxicity/Carcinogenicity (August 1998)

Group D—Genetic Toxicity Test Guidelines

- 870.5100—Bacterial Reverse Mutation Test (August 1998)
- 870.5140—Gene Mutation in *Aspergillus nidulans* (August 1998)
- 870.5195—Mouse Biochemical Specific Locus Test (August 1998)
- 870.5200—Mouse Visible Specific Locus Test (August 1998)
- 870.5250—Gene Mutation in *Neurospora crassa* (August 1998)
- 870.5275—Sex-Linked Recessive Lethal Test in *Drosophila melanogaster* (August 1998)
- 870.5300—In vitro Mammalian Cell Gene Mutation Test (August 1998)
- 870.5375—In vitro Mammalian Chromosome Aberration Test (August 1998)
- 870.5380—Mammalian Spermatogonial Chromosomal Aberration Test (August 1998)
- 870.5385—Mammalian Bone Marrow Chromosomal Aberration Test (August 1998)
- 870.5395—Mammalian Erythrocyte Micronucleus Test (August 1998)
- 870.5450—Rodent Dominant Lethal Assay (August 1998)
- 870.5460—Rodent Heritable Translocation Assays (August 1998)
- 870.5500—Bacterial DNA Damage or Repair Tests (August 1998)
- 870.5550—Unscheduled DNA Synthesis in Mammalian Cells in Culture (August 1998)
- 870.5575—Mitotic Gene Conversion in *Saccharomyces cerevisiae* (August 1998)
- 870.5900—In vitro Sister Chromatid Exchange Assay (August 1998)
- 870.5915—In vivo Sister Chromatid Exchange Assay (August 1998)

(continued)

TABLE 23.5 (continued)
Office of Chemical Safety and Pollution Prevention
(OCSPP) Harmonized Test Guidelines

Group E—Neurotoxicity Test Guidelines

- 870.6100—Acute and 28-Day Delayed Neurotoxicity of Organophosphorus Substances (August 1998)
- 870.6200—Neurotoxicity Screening Battery (August 1998)
- 870.6300—Developmental Neurotoxicity Study (August 1998)
- 870.6500—Schedule-Controlled Operant Behavior (August 1998)
- 870.6850—Peripheral Nerve Function (August 1998)
- 870.6855—Neurophysiology Sensory Evoked Potentials (August 1998)

Group F—Special Studies Test Guidelines

- 870.7200—Companion Animal Safety (August 1998)
- 870.7485—Metabolism and Pharmacokinetics (August 1998)
- 870.7600—Dermal Penetration (August 1998)
- 870.7800—Immunotoxicity (August 1998)

Group G—Health Effects Chemical-Specific Test Guidelines

- 870.8355—Combined Chronic Toxicity/Carcinogenicity Testing of Respirable Fibrous Particles (July 2001)

Source: www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series870.htm, 2012.

Toxic Substances Control Act (TSCA)

Introduction

This section outlines parts of TSCA that relate to toxicology and will be of particular interest to toxicologists. Brief descriptions are provided indicating the role of the US EPA in the implementation of this law and the handling and use of toxicological information.

Background: TSCA Testing of Existing Chemical Substances

TSCA Section 4 relates to testing of “existing chemicals.” Existing chemicals are chemical substances that are listed on the *TSCA Chemical Substances Inventory* (often referred to as the “TSCA or EPA Inventory”). Section 4 gives the EPA authority to require toxicological testing of selected substances, or mixtures containing one or more of those substances, to clarify or substantiate potential health or environmental hazards and risks. Testing may be required if the EPA believes that a substance may present an unreasonable risk of injury to health or the environment or if there may be significant exposure because of potential for use and/or the quantity produced or released into the environment. (See 56 Federal Register 32294, July 15, 1991, for details.⁷)

Although several federal agencies and EPA program offices can request the EPA to consider Section 4 test rules for chemical substances, the main source of such a request

or recommendation is the Interagency Testing Committee (ITC). The ITC is an advisory committee directed by TSCA to establish testing priorities for TSCA-regulatable chemical substances and mixtures. It also recommends the kinds of tests that should be performed under TSCA Section 4. In making a recommendation, the ITC considers all available data on and potential for carcinogenic, mutagenic, teratogenic, and chronic toxic effects, as well as a substance’s ability to bioaccumulate or cause adverse environmental effects. The ITC maintains a “TSCA Section 4(e) Priority Testing List” and transmits revisions of the list in the form of a “report” to the EPA administrator every 6 months for EPA action and publication in the Federal Register.

The EPA can issue a Section 4 test rule, arrange for testing by way of a consent agreement with industry manufacturers, or determine that additional testing is not necessary. The types of testing generally associated with Section 4 test rules fall into the following three categories:

1. Health effects testing: includes general toxicity, exposure assessment, specific organ/tissue toxicity, neurotoxicity, and/or metabolism
2. Environmental effects testing: includes aquatic and terrestrial toxicity testing
3. Chemical fate testing: includes the determination of selected physical and chemical properties, transport processes, as well as testing to determine transformation processes

Test data may be developed following the OCSPP harmonized test guidelines (Table 23.5) or Organization for Economic Cooperation and Development (OECD) testing. All studies conducted by industry under TSCA Section 4 must be performed in accordance with established test methods (“guidelines”) and must adhere strictly to EPA’s Good Laboratory Practice (GLP) Standards regulations (see 40 CFR Part 792).

TSCA Section 5: Premanufacturing Notification

TSCA Section 5 requires that a manufacturer (or importer) submit a premanufacturing notification (PMN) or one of the several possible exemption applications to the EPA before a substance is manufactured or imported for commercial purposes in the United States (if the substance is not on the *TSCA Chemical Substance Inventory*, also referred to as the “TSCA or EPA Inventory”). There are exemptions to this requirement that allow a manufacturer or importer to handle, process, and conduct research and development (R & D); test market; and even produce in low volume for commercial purposes (see CFR, Title 40, Part 720, Sections 30, 36, and 38, and Part 723, Sections 50, 175, and 250).¹ Some exemptions require prior EPA notification and approval, while others do not. In all cases, detailed recordkeeping is required to comply with TSCA Section 5.

Section 5 of TSCA does not require that toxicological testing be performed before a PMN submittal (unless the PMN substance is subject to a Section 4 test rule). However, if the manufacturer or importer has toxicological data, it must be included with the PMN submittal. If the PMN substance is subject to a TSCA Section 4 test rule, the submitter must submit the test data specified in the rule as part of the PMN. The EPA reports that only about 50% of the PMNs filed are accompanied by toxicological test data.

The EPA has 90 days to review the PMN to determine whether or not manufacture and use of the PMN substance is likely to present an unreasonable risk of injury to health or the environment. On completion of the review, the EPA may allow the substance to be manufactured or imported, it may prohibit manufacture or importation, or it may impose restrictions or conditions to which the manufacturer or importer must adhere. The EPA can also discontinue or extend the 90-day review period if there is insufficient toxicological or exposure information available for the EPA scientists to make a risk assessment. In this case, a submitter may obtain the needed additional information so that the EPA can continue the review, the submitter can perform toxicological testing under a Section 5(e) consent order while manufacturing with restrictions, or the submitter may elect not to perform the testing requested by the EPA and simply withdraw the PMN that will end the PMN review process and the substance cannot be placed into commerce.

Any scientifically valid test protocol used to generate toxicological data is acceptable to the EPA as long as it is reliable and is conducted in accordance with GLP Standards. (See 40 CFR 792.⁸) The submitter will need

to verify to the EPA that the test protocol is valid and that GLPs were followed.

TSCA Section 8(c): Allegations of Significant Adverse Reactions

TSCA Section 8(c) requires that manufacturers, processors, and distributors of chemical substances and mixtures maintain records as to allegations that chemical substances might cause significant adverse reactions to health or the environment. It is primarily a recordkeeping rule, whereas TSCA Sections 8(e) and 8(d) are reporting rules. Employee health-related allegations arising from any employment-related exposure must be retained for 30 years. Any other record of alleged significant adverse reactions must be retained for 5 years. Examples of “other” records include allegations of human health effects (listed in Table 23.6), suffered by non-employees, or environmental effects alleged by employees or nonemployees. Section 8(c) rule exempts “known” human effects as described in the scientific literature, Material Safety Data Sheets, or on a product label. However, an effect is not a “known human effect” if

1. It is a significantly *more severe* effect than described previously.
2. It is a manifestation of a toxic effect following a significantly shorter exposure period or lower exposure level than described.
3. It is a manifestation of a toxic effect by an exposure route different from that described in public information.

TABLE 23.6
Examples of Adverse Reactions^a Subject to TSCA 8(c)

Health Effects

- Long-lasting or irreversible damage, for example, cancer or birth defects
- Partial or complete impairment of bodily functions, for example, reproductive disorders, neurological disorders, or blood disorders
- An impairment of *normal activities* experienced by all or most of the persons exposed at one time
- An impairment of normal activities that is experienced *each time* an individual is exposed

Environmental Effects

- Gradual or sudden changes in the composition of animal or plant life in an area (including fungal or microbial organisms)
- Abnormal number of deaths of organisms (e.g., fish kills)
- Reduction of the reproductive success or the vigor of a species
- Reduction in agricultural productivity, whether crops or livestock
- Alterations in the behavior or distribution of a species
- Long-lasting or irreversible contamination of components of the physical environment, especially in the case of groundwater, surface water, and soil resources that have limited self-cleansing capability

^a Significant adverse reactions are reactions that *may* indicate a substantial impairment of normal activities or long-lasting or irreversible damage to health or the environment.

TSCA Section 8(d): Reporting of Health and Safety Studies

Section 8(d) of TSCA requires that manufacturers (including importers) of specified chemical substances (or mixtures containing those substances) submit unpublished health or environmentally related studies in their possession to the EPA. Such submittals may include epidemiological data, occupational/environmental exposure data, or health/environmental toxicology studies. The TSCA Section 8(d) regulations with specific reporting requirements are defined in the CFR, Title 40, Part 716.⁸ TSCA defines a health and safety study as being any study of any effect of a chemical substance on health or the environment. The specific substances as well as certain categories of substances that are or have been subject to TSCA Section 8(d) in the past can be found in the CFR, Title 40, Part 716, Section 120.⁸ The final rule providing details of the TSCA Section 8(d) reporting requirements was published in the 9-15-86 *FR*, Volume 51, page 32,720.⁹

A manufacturer or processor who intends to conduct a new study relating to a chemical substance on the 8(d) list must submit a notice of initiation to the EPA when the study is started and the final report must be submitted to the EPA upon completion of the study. However, findings that constitute “substantial risk” information under Section 8(e) must be reported to the EPA within 15 working days of its receipt. This means that it might be necessary to report *some* observations to EPA even as the test is in progress.

Types of information typically reported and information that is not reportable under TSCA Section 8 (d) are summarized in Tables 23.7 and 23.8.

TABLE 23.7

Examples of Information Typically Reported under TSCA Section 8(d)

- Mutagenicity, carcinogenicity, or teratogenicity data
- Studies indicating potential for neurotoxicity or behavioral disorders
- Studies of sensitization, pharmacological effects, mammalian absorption/distribution/metabolism/excretion, cumulative/additive/synergistic effects, and acute/subchronic/chronic health or environmental effects
- Test data relating to ecological/environmental effects on invertebrates, fish, or other aquatic animals/plants
- Assessments of human or environmental exposure including workplace exposure data where the data are analyzed with respect to potential health or environmental effects
- Monitoring data when analyzed to determine the degree of exposure of a listed 8(d) substance to humans or the environment and related effects

TABLE 23.8

Examples of Information Not Reportable under TSCA 8(d)

- Studies that have been published in the scientific literature (TSCA is looking for “unpublished” data)
- Data previously submitted to the EPA under Sections 8(e), 4 or 5, or for EPA’s information (FYI submissions)
- Data relating to substances that are *not* listed on EPA’s Section 8(d) list (see 40 CFR 716.120)
- Monitoring data (workplace or environmental) that were collected and analyzed more than 5 years before the substance was placed on the 8(d) list.
- Data previously submitted to the EPA under TSCA Section 8(d) by trade associations on behalf of member companies

TSCA Section 8(e): Substantial Risk Notification

Section 8(e) of TSCA requires that any person who manufactures, imports, processes, or distributes in commerce a chemical substance or mixture of substances and who obtains information that “reasonably” supports the conclusion that the substance or mixture presents a substantial risk of injury to health or the environment must inform the EPA administrator of such information (unless it is known that the EPA administrator already has knowledge of the information).

The TSCA 8(e) reporting requirements became effective on January 1, 1977, and the EPA issued a proposed policy statement on September 9, 1977 (42 FR 45362).¹⁰ A “Statement of Interpretation and Enforcement Policy; Notification of Substantial Risk,” describing the types of information subject to TSCA 8(e) reporting and the procedures for doing so, was published in the Federal Register on March 16, 1978 (43 FR 11110).¹¹

Section 8(e) of TSCA is an information-gathering tool directed at new-found serious chemical hazards and/or exposures:

1. Information that meets the reporting criteria *need not* establish *conclusively* that a substantial risk actually exists. Key criteria to be considered when determining whether new-found information is reportable or not are the seriousness of the adverse effect, the potential for human or environmental exposure to the substance or mixture, and whether the information is already known by the EPA administrator or not.
2. Substances *not* subject to TSCA 8(e) reporting requirements (exempt by definition) are
 - a. Pesticides that are in commerce solely for use as pesticides. A chemical substance that is in the process of research and development (R&D) as a pesticide *is* subject to TSCA 8(e) reporting *until* an application for an “Experimental Use Permit” (EUP) is submitted to the EPA or the substance/mixture is registered under FIFRA.

- b. Tobacco or tobacco products.
- c. Source materials, special nuclear materials, and by-products (as defined in the 1954 Atomic Energy Act and relevant regulations).
- d. Foods, food additives, drugs, cosmetics, and devices (as defined in the Federal Food, Drug and Cosmetics Act [FFDCA]) when manufactured, imported, processed, or distributed in US commerce as a food, food additive, drug, cosmetic, or device.

All chemical substances (except those exempted by definition) including but not limited to R & D chemical substances, laboratory reagents, polymers, intermediates (whether isolated or not!), catalysts by-products, impurities, and TSCA-covered microorganisms and products therefrom are subject to the TSCA 8(e) reporting requirements.

Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)

Introduction

Pesticides are used to control unwanted insects, fungi, weeds, microbes, and rodents. They are intended to protect human health from diseases or toxins, to increase the yield of crops, and to improve the natural beauty of the environment. For example, insecticides can be used to control the following: insects that transmit bacterial or viral diseases or allergens to humans or plants; insects that directly eat crops or parts of plants, shrubs, or trees; termites, carpenter and fire ants, and cockroaches; and grubs and worms that feed on the roots of turf and field crops. Fungicides are used to prevent the destruction of plants, trees, or grasses and to keep crops untainted by mycotoxins. Herbicides act by selectively killing weeds that compete with crops for nutrients in soil and water and thereby can indirectly increase crop yields. Herbicides are also used on turf to improve the aesthetics of lawns around the home and golf courses and for total vegetation control to eliminate unwanted vegetation around railroad tracks, rights of way, fences, etc. Algicides are used aquatically to improve water flow, which in turn may prevent flooding. Antimicrobial agents are used to kill bacteria and viruses and thereby limit the spread of infectious agents in the home, hospitals, restaurants, and food-processing establishments. Finally, rodenticides are intended to kill rodents that spread diseases and excrement in food-storage areas or in urban areas where garbage is concentrated.

Fundamentally, pesticides are medicines developed to treat diseases and to prevent both the spread of disease and predator destruction of trees, shrubs, grasses, and crops. As a result, pesticides prevent the vector-borne spread of diseases to humans and plants and increase crop yields, which help provide an inexpensive source of fruits and vegetables necessary for maintaining optimum human health. Further, antifungal

agents prevent fungal contamination of agricultural crops with mycotoxins that can be extremely toxic and, in some cases, carcinogenic to humans.

The original legislation, entitled FIFRA, initially assigned labeling authority of pesticides to the US Department of Agriculture (USDA). The most significant amendment to FIFRA occurred in 1972; the amendment transferred most regulatory authority of pesticides to the newly formed (1970) EPA. Under this amendment, pesticides would be registered as either general-use or restricted-use pesticides, the latter requiring handling by a *certified* pesticide handler only, who would be certified by the states. This amendment also required that pesticides not only be registered with the EPA but also be reregistered with the EPA, assuring that reevaluation of the safety of a pesticide would be performed periodically using up-to-date safety standards. Additional federal actions since 1972, with the most recent act entitled the "Food Quality Protection Act" (FQPA) (1996) and Pesticide Registration Improvement Act (PRIA) (2003), are all intended to expand the requirements and standards for registering and reregistering pesticides to protect humans, especially children, and the environment by minimizing the level of risk in the use of pesticides, that is, by providing a standard of "reasonable certainty of no harm."

Office of Pesticide Programs

The Office of Pesticide Programs (OPP), which regulates the pesticide industry under FIFRA, is within the OCSPP, formerly the Office of Prevention, Pesticides and Toxic Substances (OPPTS). The Health Effects Division requires that mammalian toxicology testing be performed to evaluate the risk to humans from pesticide use. The Environmental Fate and Effects Division requires ecological toxicology testing and environmental fate analyses to determine potential risks to birds, fish, wildlife, invertebrates, and nontarget plants in both terrestrial and aquatic environments.

Testing Requirements

For pesticides, whose general-use patterns include terrestrial, aquatic, and greenhouse uses for both food crops and non-food, as well as forestry, domestic outdoor, and indoor uses for nonfood, a broad selection of toxicology studies is used to screen each chemical for the purposes of dietary, worker, and nonworker risk assessments. These requirements for testing were proposed in the Federal Register (October 24, 1984) and are now listed in 40 CFR 158.340.

Although acute and short-term studies may be conducted by various routes of exposure, for example, dermal, inhalation, and oral intubation (gavage), longer-term studies are generally performed by administering the pesticide in the diet, which is consistent with general long-term exposure of humans to pesticide residues in the food. However, these pesticides are tested to a maximum tolerated dose or a limit dose of 1,000 mg/kg body weight/day (approximately 20,000 ppm

in the rat [S-D] and 7,000 ppm in the mouse [CD-1]), whereas most foods have no detectable pesticide residues and, when present, are often found only in ppb ranges.

For certain classes of chemicals, such as pesticides (usually insecticides) with neurotoxic activity, the OPP (Health Effects Division) at the EPA requires for new registrations or reregistration, both acute and subchronic (90-day) neurotoxicity studies in rats and, for organophosphate cholinesterase inhibitors, acute and 28-day delayed neurotoxicity studies in hens, including measurements of neuropathy target esterase. Other conditionally required studies, which may be requested depending on results (triggers) from the previously mentioned studies or on specific-use patterns, include the following studies in rats: subchronic (90-day) dermal or inhalation studies (particularly for indoor residential exposures), chronic neurotoxicity studies, dermal penetration studies, and immunotoxicity studies. Following the passage of the 1996 FQPA, which requires evaluation of potential increased sensitivity of infants and children, the EPA requested that the developmental neurotoxicity study in rats be performed for pesticides showing neurotoxic activity and, more recently, subtle disruption of the endocrine system.

Some of the previously mentioned studies may be waived depending on the use pattern of the pesticide. For example, a pesticide used only on golf courses may have the chronic/oncogenicity studies and reproduction studies waived and a 90-day dermal toxicity study substituted for the subchronic feeding study in rats. It should also be noted that the acute battery of six studies and occasionally the 21/28-day dermal toxicity studies are required for registration, not only of the active ingredient but also of all end-use formulations, unless waivers for these studies are granted by the EPA.

Finally, there is a class of pesticides referred to as biopesticides and microbial pest control agents (MPCAs). These include pheromones and other naturally occurring compounds, bacteria, algae, fungi, viruses, protozoa, and plant pesticides, as defined in 40 CRF 152.20. Biological and biologically derived pesticides are generally naturally occurring or strain improved, either by natural selection or by deliberate genetic manipulation.

Because biopesticides are specific to the target species and typically have a unique or nontoxic mode of action, the EPA published in 1983, and amended in 1989, testing guidelines classified as subdivision M of the Pesticide Assessment Guidelines. The testing requirements are a tiered system; the first tier consists of a battery of short-term tests designed to evaluate potential toxicity, infectivity, and pathogenicity of the biopesticide (OCSPS Series 885, Group C). Both the design and extent of testing are determined on a case-by-case basis by the EPA to minimize unnecessary testing but to assure that sufficient testing is performed to make scientifically sound regulatory decisions. Ecological and nontarget organism and environmental expression testing (OCSPS Series 885, Groups D and E) also have been grouped in tiers.

Typical toxicology studies required for pesticides are listed in Table 23.9.

TABLE 23.9

Typical Toxicology Studies Required for Pesticides^a

- Six acute studies—rat oral, dermal, inhalation, and primary eye (rabbit) and skin (rat) irritation, and dermal sensitization (guinea pig or local lymph node assay (LLNA) in mice)
- 21/28-day dermal toxicity study
- Subchronic (90-day) feeding studies in rodents and nonrodents (e.g., dogs)
- Chronic feeding studies in rodents and nonrodents
- Oncogenicity studies in two species of rodents (rat and mice preferred)
- Prenatal developmental toxicity studies in rodents and nonrodents (rats and rabbits preferred)
- Two-generation reproduction study in rodents
- General metabolism/pharmacokinetic study in rodents (usually rats)
- Genotoxicity studies—in vitro gene mutation (bacterial and mammalian cells), structural chromosomal aberration (e.g., rodent, in vivo), and other genotoxic effects

^a Additional studies may be required or some of the previously mentioned studies waived on a case-by-case basis (see text).

EUROPEAN CHEMICAL REGULATION

REACH: European Union

Previous legislation concerning registering of new chemicals and associated toxicity testing requirements was replaced in 2007 by the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). This all-encompassing legislation establishes a framework for procedures and documentation required for manufacture and distribution of chemicals in the EU. Manufacturers and importers of chemicals in the EU are required to register all substances to be placed on the market with requirements linked to quantities of substances marketed with yearly production and cumulative thresholds. Evaluations of two types are performed, (1) dossier evaluation and (2) substance evaluation, and result in either restriction or authorization. Dossier evaluation consists of review of existing data. Data sharing among manufacturers is mandatory. Substance evaluation is performed by the ECHA (European Chemicals Agency) (website: <http://echa.europa.eu/chemicals-in-our-life>). A major focus of REACH legislation is to avoid animal testing to the maximum extent possible and to determine if animal testing is truly necessary. This legislation mandates evaluation of all other possible sources of information and recommends animal testing, which is to be conducted “only as a last resort.”

PHARMACEUTICALS

Chapter 20 of this handbook, Pharmaceutical Toxicology, presents an extensive discussion of regulatory requirements for the development of pharmaceuticals in the United States, under the Food and Drug Administration (FDA); in the EU, under the European Medicines Agency (EMA); and in Japan, under the Ministry of Health, Labor and Welfare

(MHLW), and for agencies in other countries. Most agencies that regulate pharmaceuticals perform safety testing following guidances established by the International Conference on Harmonization (ICH). A list of safety guidances is presented in Table 23.10.

TABLE 23.10
International Conference on Harmonization (ICH)
Final Safety Guidances

Number	Title	Date
Carcinogenicity Studies		
S1A	The Need for Long-Term Rodent Carcinogenicity Studies of Pharmaceuticals	3/1/96
S1B	Testing for Carcinogenicity of Pharmaceuticals	2/28/98
S1C(R2)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals	9/17/08
Genotoxicity Studies		
S2A	Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	4/1/96
S2B	Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals	11/21/97
S2(R1)	Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use	6/6/12
Toxicokinetics and Pharmacokinetics		
S3A	Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies	3/1/95
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies	3/1/95
Toxicity Testing		
S4A	Duration of Chronic Toxicity Testing in Animals (Rodent and Nonrodent Toxicity Testing)	6/25/99
M3(R2)	Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals	1/20/10
Reproductive Toxicology		
S5A	Detection of Toxicity to Reproduction for Medicinal Products	11/05
S5(R2)	Detection of Toxicity to Reproduction for Medicinal Products—Toxicity to Male Fertility	11/05
Biotechnology-Derived Pharmaceuticals		
S6(R1)	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals	5/17/12
Safety Pharmacology Studies		
S7A	Safety Pharmacology Studies for Human Pharmaceuticals	7/1/01
S7B	Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals	10/19/05
Immunotoxicity Studies		
S8	Immunotoxicity Studies for Human Pharmaceuticals	4/12/06
Anticancer Pharmaceuticals Evaluation		
S9	Nonclinical Evaluation for Anticancer Pharmaceuticals	3/5/10

MEDICAL DEVICES

INTRODUCTION

Although the use of medical devices has a long history, the modern medical use of devices only became realistic as an effective therapeutic intervention as the use of aseptic techniques expanded in the late 1800s. The first devices were, of course, fashioned from materials found in nature. Often, materials were obtained from domesticated animals. For example, leather was sculptured to form replacements for lost ears and noses, and goose trachea was used as tubing in the measurement of arterial pressure. In all cases, the key to the search for successful device materials was identifying materials with appropriate mechanical properties while minimizing the potential for adverse biological reactions.

Although tissues obtained from domestic animals continue to be used for medical devices, material science has continued to develop and now includes many forms of metal, ceramic, plastics, and other synthetic polymers. The role of toxicology in medical device development is to ensure that the materials selected for use in devices are safe for their intended use. As in all disciplines of toxicology, the safety tests used to evaluate a device material must be appropriate to the route and duration of exposure. This section provides information, related to both testing and regulatory strategies, intended to be beneficial to the medical device researcher.

Even though the difference between a medical device and a drug may seem obvious, the clarity of the distinction is becoming blurred as therapeutic interventions become more complex and disease specific. The difference between a drug and a device can generally be summarized by the simple observation that a drug is independent of its physical form as long as the physical form allows for systemic absorption of the active drug. However, a medical device is generally dependent upon its physical form to exert its therapeutic (biologic) effect. There are multiple examples of therapeutic medicaments that are not easily classified as either a drug or a device based upon their mechanism of action and intended therapeutic effect. The specific issues associated with the categorization of a treatment modality as either a device or drug are beyond the scope of this section.

The development of the European Community and the publication of the International Standards Organization (ISO) guideline documents have had significant impact on the medical device manufacturer. The regulatory arena continues to evolve and the information in this section presents a snapshot of the landscape.

HISTORICAL PERSPECTIVE

Because of the difficulties encountered when attempting to evaluate the safety of a solid test article, a historical description of the development of medical devices is considered useful to anyone evaluating medical devices for safety. The inherent safety of “natural” materials was

often assumed. This, of course, was not always supported under use conditions. Synthetic materials originally selected for medical use were chosen primarily for their physical attributes. These were materials first created for military, commercial, or industrial purposes. Since these materials were essentially considered to be inert and stable in the biological environment, the safety of medical device materials initially focused on sterility. However, as the developing device industry evolved and the devices were intended to treat increasingly complex conditions, materials were increasingly scrutinized for appropriateness for use in the biological environment.

As researchers examined the biological effects of medical device materials, whether synthetic or natural, under use conditions, adverse events were increasingly noted.¹² Therefore, a need for methods to determine the safety of materials was recognized by both the medical device industry and regulatory agencies, and the focus of medical device material identification shifted from purely performance to a combination of performance and safety. To that end, the industry became aware that a procedure was necessary for predicting which materials could be used safely.¹⁴

Since plastics were already a common material used in many varied devices, a set of tests designed by the US Pharmacopeia (USP) for classifying plastics using a series of biological tests was selected as a guidance.¹⁴ In this classification system, the proposed end use of the plastic, including the nature and extent of expected biological interaction, as well as possible solutions (e.g., drug vehicles) that may contact the plastic, is used as a guide to determine for which class the plastic should be qualified. Then, to determine whether the plastic is suitable for that class, a series of biological reactivity tests must be conducted. Representative samples of the plastic are used for the biological reactivity testing using specific methods prescribed by the USP. If the results of the tests are acceptable, as defined by the USP, for all the tests in the selected class, the plastic is considered to have met the requirements for that class (Table 23.11 presents a summary of the tests to be successfully conducted for each class). It is important to note that the class into which a plastic is placed is not related to its physical form or chemistry but is defined by the tests conducted and successfully completed.

Although the classification of plastics by the USP methods is not generally considered to address adequately the safety of medical devices, a knowledge of the classes is important for the material researcher since some plastic raw materials may be identified using a plastic classification. This classification may therefore provide the researcher with a baseline indication of the appropriateness of the raw material for possible use in a device. It is important to note that the plastic class does not indicate safety of the plastic raw material in the final device and it is the responsibility of the device manufacturer to substantiate safety of the final device utilizing appropriate test methods. To that end, in 1976, the US Congress

passed the Medical Device Amendments to the Food, Drug, and Cosmetic Act. The critical wording of this act necessitated an emphasis on the nature of the end use of a medical device in the assessment of safety parameters. Furthermore, the focus of safety testing was the final device, as intended for use, rather than individual raw materials to be used in the device.

In part to address the requirements of this act, the US FDA, in collaboration with Canada and Great Britain, drafted a guidance document in 1987 for medical devices, known as the Tripartite Agreement. This agreement utilized a matrix approach of intimacy and duration of tissue contact to categorize a medical device based upon its intended use. This assumed that more critical medical devices (i.e., those implanted into the body, life supporting, and/or life sustaining) would, by definition, have a greater intimacy of tissue contact and a longer duration of that contact.⁴ Based upon the category determined by the parameters of the tripartite matrix, the necessary safety evaluations that had to be successfully completed before the device could be marketed were identified.

The intended use of a device, specifically the intimacy of tissue contact combined with the duration of this contact, as initially defined by the Tripartite Agreement would dominate the selection of safety tests for a specific device both then and now. However, other criteria for categorization of medical devices have been recognized, including whether the device has direct or indirect contact with biological tissues (see in the following text), and today considerations for medical device testing may include inductive and inflammatory responses, general toxicity, specific organ toxicity, immunotoxicity, reproductive toxicity, genetic toxicity, and carcinogenicity.

The Tripartite Agreement has been replaced by a more international series of guidance documents that continue to be prepared and refined. User fee legislation has also been enacted in the United States by the FDA in an attempt to accelerate review of applications. The ISO has led the current efforts to establish a series of international medical device guidelines. The ISO guidance documents maintained the matrix approach of the Tripartite Agreement and proposed a guidance document in 1992. The focal point of the guidance was ISO 10993-1 Biological Evaluation of Medical Devices—Part 1: Guidance on Selection of Tests. Additional supporting documents have been and continue to be developed. The titles of other parts of the ISO 10993 guidance (current through 2007)¹⁶ are shown in Table 23.12, although not all guidance documents are finalized. The ISO 10993 guidance documents have become the standard for manufacturers desiring to market medical devices in Europe, Japan, and the United States. The ISO 10993 series of documents were formally adopted by the FDA, with modification, in 1995. However, the ISO 10993 guidance documents are dynamic, and the reader is encouraged to ensure that the most up-to-date information is available before initiating any testing.

TABLE 23.11
Classification of Plastics^a

Class I

- *Saline extract* of sample evaluated using the systemic injection and intracutaneous reactivity tests

Class II

- *Saline extract* of sample evaluated using the systemic injection and intracutaneous reactivity tests
- *Ethyl alcohol in saline (5% v/v) extract* evaluated using the systemic injection and intracutaneous reactivity tests

Class III

- *Saline extract* of sample evaluated using the systemic injection and intracutaneous reactivity tests
- *Ethyl alcohol in saline (5% v/v) extract* evaluated using the systemic injection and intracutaneous reactivity tests
- *PEG 400 extract of sample* evaluated using the systemic injection test (intraperitoneal [ip])
- *Vegetable oil extract of sample* evaluated using the systemic injection test (ip)

Class IV

- *Saline extract* of sample evaluated using the systemic injection and intracutaneous reactivity tests
- *Ethyl alcohol in saline (5% v/v) extract* evaluated using the systemic injection and intracutaneous reactivity tests
- *Vegetable oil extract of sample* evaluated using the systemic injection (ip) and intracutaneous reactivity tests
- *Sample (not extract)* implanted into the muscle of an animal and evaluated for macroscopically visible signs of inflammation

Class V

- *Saline extract* of sample evaluated using the systemic injection and intracutaneous reactivity tests
- *Ethyl alcohol in saline (5% v/v) extract* evaluated using the systemic injection and intracutaneous reactivity tests
- *PEG 400 extract of sample* evaluated using the systemic injection (ip) and intracutaneous reactivity tests
- *Vegetable oil extract of sample* evaluated using the systemic injection (ip) and intracutaneous reactivity tests

Class VI

- *Saline extract* of sample evaluated using the systemic injection and intracutaneous reactivity tests
- *Ethyl alcohol in saline (5% v/v) extract* evaluated using the systemic injection and intracutaneous reactivity tests
- *PEG 400 extract of sample* evaluated using the systemic injection (ip) and intracutaneous reactivity tests
- *Vegetable oil extract of sample* evaluated using the systemic injection (ip) and intracutaneous reactivity tests

Source: US Pharmacopeia 32/National Formulary 27, April 30, 2010 edition, United States Pharmacopeial Convention, Inc., Rockville, MD, 2010, Chapter 88, p. 99.

Note: All systemic tests are dosed by the intravenous route of administration unless noted in the table as being dosed by the (ip) route of administration. PEG 400 = polyethylene glycol 400, which may be diluted with saline prior to conducting the biological assay.

^a If a plastic raw material is considered to meet the requirements of one of these classes, testing of a representative sample of that plastic would have met the acceptable standards for all of the very specific, biological reactivity tests listed below that class. See the USP, 1994, for the specific testing parameters and criteria for meeting the individual test procedures.

TABLE 23.12
ISO 10993—Biological Evaluation of Medical Devices

Part	Title
1	Evaluation and testing Evaluation and testing within a risk management system
2	Animal welfare requirements
3	Tests for genotoxicity, carcinogenicity, and reproductive toxicity
4	Selection of tests for interaction with blood (and Amendment 1)
5	Tests for <i>in vitro</i> cytotoxicity
6	Tests for local effects after implantation
7	Ethylene oxide sterilization residuals
9	Framework for identification and quantification of potential degradation products
10	Tests for irritation and delayed-type hypersensitization (and Amendment 1)
11	Tests for systemic toxicity
12	Sample preparation and reference materials
13	Identification and quantification of degradation products from polymeric medical devices
14	Identification and quantification of degradation products from ceramics
15	Identification and quantification of degradation products from metals and alloys
16	Toxicokinetic study design for degradation products and leachables
17	Establishment of allowable limits for leachable substance
18	Chemical characterization of materials
19	Physicochemical, morphological, and topographical characterization of materials
20	Principles and methods for immunotoxicology testing of medical devices

REGULATION OF MEDICAL DEVICES: UNITED STATES

There are several methods of classification of devices in the United States. The method used will depend upon the specific use for the device and, importantly, by whom and how the device is intended to be used. The FDA has established specific device categories based upon medical specialties (Table 23.13). The FDA has further assigned each of these generic classes of devices to one of three regulatory classes (Table 23.14). The assignment of a regulatory class is based upon the level of control the FDA considers to be necessary to ensure the safety and effectiveness of the device when manufactured and marketed as intended.

To market a device in the United States, the manufacturer must determine whether a regulatory submission is necessary. Generally, a submission to FDA will be necessary to market a medical device in the United States unless the FDA has published a notice indicating that this generic class of device is exempt from the premarket notification

procedure. Devices exempted by FDA for submission are generally class I devices for which the FDA considers general manufacturing controls to be sufficient to ensure the safety and efficacy of the device for the patient and/or user.

When it has been determined that a submission to FDA is necessary prior to marketing a device in the United States, the manufacturer must then determine the type of submission. In the United States, the two forms of submission are the premarket notification, or 510(k), and the premarket approval (PMA). Most devices on the market in the United States are class II, and therefore, most devices are cleared for commercial distribution in the United States by the 510(k) process. Most class I devices are exempt from the need for a regulatory submission, and most class III devices require a PMA application (see in the following text) prior to marketing in the United States. Although it is beyond the scope of this section to describe the specific issues associated with these submissions, a brief description of each is provided.

Areas that generally must be addressed in a regulatory submission required for either the 510(k) or PMA are shown in Table 23.15.

The 510(k) is a submission to the FDA demonstrating that the proposed noncritical and nonexempt device (generally class II) is as safe and as effective (i.e., substantially equivalent) to a legally marketed device that was or is currently in the US market. Therefore, an important aspect of the 510(k) process is identification of the previously marketed, substantially equivalent device for use in comparison with the proposed device. The substantial equivalence of the marketed and proposed device is established based upon a number of factors, but the two devices do not have to be identical. The need for toxicological/safety testing will be, at least partially, based on the similarity of the two devices including materials and manufacturing practices. Clearly, the manufacturer must provide, at the least, adequate safety information on any new materials in the new device.

The PMA process is used by the FDA to evaluate the safety and effectiveness of noncritical and nonexempt devices (usually class II) for which a currently marketed, substantially equivalent device cannot be identified as well as most critical devices (class III). Because of the level of risk associated with the use of critical devices, the FDA determines that these devices require a rigorous regulatory submission prior to marketing. To substantiate safety and efficacy, the PMA process requires more information than what is presented during a comparison to a preexisting device. Therefore, each critical device marketed in the United States must be individually shown to be safe and effective.

TABLE 23.13
FDA Device Categories (21 CFR 860)

862	Clinical Chemistry and Clinical Toxicology
864	Hematology and Pathology
866	Immunology and Microbiology
868	Anesthesia
870	Cardiovascular
872	Dental
874	ENT
876	Gastroenterology–Urology
878	General and Plastic Surgery
880	General Hospital and Personal Use
882	Neurology
884	Obstetrics–Gynecology
886	Ophthalmology
888	Orthopedics
890	Physical Medicine
892	Radiology

TABLE 23.14
Three Regulatory Classes—United States

Class I. General Controls (with or without Exemptions)
Least perceived risk to device user and/or patient
Elastic bandages
Stethoscope
Manual surgical instruments (e.g., scalpels, tissue retractors)
Oxygen mask
Canes and crutches
Class II. General Controls and Performance Standards (with or without Exemptions)
Mercury thermometer
Diagnostic test
Cardiac catheter
Condom
Blood pressure cuff
Class III. General Controls and PMA
Most perceived risk to device user and/or patient
Pacemaker
Implanted neuromuscular stimulator (e.g., defibrillator)
Replacement heart valve
Computerized microscopes that automatically read Pap smears

TABLE 23.15
Areas to Be Addressed in a Regulatory Submission to the FDA

Intended use
Indications for use
Target population
Design
Materials
Performance
Sterility
Biocompatibility
Mechanical safety
Chemical safety
Anatomical sites
Human factors
Energy used and/or delivered
Compatibility with the environment and other devices
Where used
Standards met
Electrical safety
Thermal safety
Radiation safety

Notes: For 510(k), to market a noncritical and nonexempt device (generally class II). Selection of predicate device that is as safe and as effective (i.e., substantially equivalent) to a legally marketed device that was or is currently on the US market.

REGULATION OF MEDICAL DEVICES: EUROPE

In Europe, the 15 original member states (along with several additional member states) of the European Community have authored, reviewed, and accepted a common method by which manufacturers must evaluate and qualify their proposed devices prior to marketing. The Active Implantable Medical Device Directive and the Medical Device Directive define the legal framework accepted by the member states through which medical devices are regulated. Through the directives and system of notified and competent bodies, many devices must meet essential requirements prior to being marketed. As in the United States, devices must be evaluated for safety and efficacy to ensure that they do not harm the patient, clinician, or any third party. Also similar to the United States, there is a system of classification determined by the Medical Device Directives as shown, with examples, in Table 23.16.

For most medical devices, the independent Notified Body often signifies that the device may be marketed in the European Community by granting the manufacturer the right to label the product or its packaging with a “CE marking,” which signifies that the manufacturer has adequately proved the safety and efficacy of the proposed device. In theory, a CE marking obtained through a Notified Body appointed by the government agency of one of the member states gives the manufacturer the right to market the subject device in any one of the member states. However, any of the individual member states may, at any time, implement a safeguard clause and

TABLE 23.16
European Classifications as Defined by the Active Implantable Medical Device (AIMD) and the Medical Device Directives (MDD)

- Placement of a device into a class is based upon risk and duration of use
- The intended use determines the class placement
- For combination devices and devices with accessories, each part of the device must be placed into a class and the highest class placement applies to the entire device
- There is no connection between the MDD classifications and FDA classifications

Class I—Low Risk
Most basic classification for medical devices
Wheelchairs
Surgical instruments

Class IIa—Low–Medium Risk
Short-term surgically invasive devices
Some active, noninvasive devices
Electrocardiographs
Hearing aids

Class IIb—Medium–High Risk
Energy/substance delivering
Utilizes or emits ionizing radiation
Long-term surgically invasive devices
Infusion pumps
Ventilators

Class III—High Risk
Most cardiovascular and CNS devices

require additional testing to evaluate the device further to ensure that the CE marking was not incorrectly granted.

DETERMINING TOXICOLOGY TESTING NEEDS

Once toxicology testing has been determined to be necessary, the specific tests to be performed must be identified by the manufacturer. The FDA and European Community both generally use the ISO 10993 Biological Evaluation of Medical Devices in the evaluation of manufacturers’ biological safety testing program for medical devices regardless of the specific regulatory submission made—510(k), PMA, or for a CE marking. As previously noted, the testing recommended by the ISO guidelines utilizes a categorization of the device based on the specific nature of the interaction of the device and component parts with the body.

The ISO standard 10993-1 as modified by the FDA (Table 23.17) summarizes the initial toxicology tests necessary for a proposed device based on the “nature of body contact,” noncontact, surface contacting (skin, mucosal membranes, and breached or compromised surfaces), external communicating (blood path, indirect; tissue/bone/dentin communicating and circulating blood), or implant devices and by the duration of contact: limited exposure (≤ 24 h), prolonged exposure (>24 h to 30 days), or permanent contact

(>30 days). Testing requirements increase as intimacy and duration of contact of the device increase.

Using the expected intimacy of contact and duration of contact for the proposed device, the intimacy and duration categories can be determined and, therefore, the required tests for biological evaluation identified. Effects on reproduction (including developmental effects) and biodegradation may be considered for specific materials/devices depending upon the intended end use. Additional tests for specific target organ toxicity such as immunotoxicity or neurotoxicity may be necessary, depending on the characteristics of the intended use of the device including the intimacy and duration of contact. Table 23.18 provides examples of some medical devices and the corresponding body contact categories.

The intended use of the device as well as the experience with and knowledge of the materials used in its manufacture must be considered during the determination of which tests to conduct. To that end, the ISO 10993 standard is intended to be used as a guide, and therefore, the final decision on which tests to be conducted must be made by individuals qualified in material biology and/or toxicology. The establishment of the safety assessment plan for a device should be made by the appropriate professionals, qualified by training and experience using interpretation and judgment, when considering the factors relevant to the device or its materials, the intended use, and current knowledge of the device and its materials provided by scientific literature and previous clinical experience.¹⁷

There are several specific situations for which ISO 10993 is *not* applicable and should not be used. Even though many of the materials used in these applications are the same, it is important that the researcher be familiar with these cases to ensure that the ISO guidance documents are not inappropriately applied to these situations. For example, packaging materials for drugs and biologics are not considered medical devices. Furthermore, several special standards for specific categories of devices are available for guidance. The researcher must ensure that the proposed device would not be included in one of these specific guidance documents before proceeding with the selection of testing methods.

For all toxicological end points, a stepwise approach to selection of tests is recommended. The researcher should first conduct a comprehensive literature review to ensure that animal studies are not inappropriately conducted. The literature search should be followed, as appropriate, by relevant *in vitro* assays and, finally, *in vivo* assays in animals if the end point has not been resolved.

In determining testing requirements for medical devices, as for other test articles to be evaluated for toxicity, it is important to consider previously conducted study results or published literature before initiating a study using animals.¹⁸ To that end, the Animal Welfare Act has defined a responsibility for the individual researcher and, therefore, the manufacturer to consider all information before initiating animal testing.

Studies often conducted during the safety evaluation of medical devices are presented in Table 23.19.

TABLE 23.17
The ISO Standard 10993-1 Guidance for Selection of Biocompatibility Tests as Modified by the FDA

Device Categories		Biological Effect ^c										
Body Contact ^a	Contact Duration ^b	Contact			Systemic		Subchronic			Chronic		
		Cytotoxicity	Sensitization	Irritation or Intracutaneous	Toxicity (Acute)	Toxicity	Genotoxicity	Implantation	Hemocompatibility	Toxicity	Carcinogenicity	
Surface devices	Skin	A	X	X	X							
		B	X	X	X							
		C	X	X	X							
	Mucosal membrane	A	X	X	X							
		B	X	X	X	O			O			
		C	X	X	X	O		X	O		O	
	Breached or compromised surfaces	A	X	X	X	O						
		B	X	X	X	O			O		O	
		C	X	X	X	O		X	O		O	
External communicating devices	Blood path, indirect	A	X	X	X	X				X		
		B	X	X	X	X				X		
		C	X	X	O	X		X	O	X	X	
	Tissue/bone/dentin communicating +	A	X	X	X	O						
		B	X	X	O	O		X	X			
		C	X	X	O	O		X	X		O	
	Circulating blood	A	X	X	X	X				X		
		B	X	X	X	X		X	O	X		
		C	X	X	X	X		X	O	X	X	
Implant devices	Tissue/bone	A	X	X	X	O						
		B	X	X	O	O		X	X			
		C	X	X	O	O		X	X	X	X	
	Blood	A	X	X	X	X						
		B	X	X	X	X		X	X			
		C	X	X	X	X		X	X	X	X	

Source: Adapted from FDA General Program Memorandum G95-1, Tables 1 and 2.

Notes: +, Tissue includes tissue fluids and subcutaneous spaces; ^, for all devices used in extracorporeal circuits.

^a See text.

^b A, limited (24 h); B, prolonged (24 h to 30 days); C, permanent (>30 days). See text.

^c x, ISO Evaluation Tests for Consideration; o, additional tests that may be applicable.

TABLE 23.18
Examples of Body Contact Categories as Determined by
the ISO 10993-1 Guidance Matrix

	Body Contact Category	Examples
Surface devices	Skin	Stethoscope head
		Electrodes
		Compression bandages
		Surgical gowns
	Mucosal membrane	Examination gloves
		Inhalers
		Endotracheal tubes
		Urinary catheters
		External feeding tubes
	Breached or compromised surface	Bandages, dressings
		Wound patches
External communicating devices	Tissue/bone/dentin communicating	Occlusive tapes
		Surgical gloves
		Laparoscopes
	Blood path indirect	Endoscopes
		Hypodermic needles
		Extension sets
		Transfer sets
		Blood transfusion sets
	Circulating blood	IV catheters
		Dialysis tubing
		Hemodialyzers
Implant devices	Tissue/bone	Oxygenator
		Bone/dental cements
		Cerebrospinal drains
		Implanted drug delivery port
		Orthopedic joints, pins, plates, screws
		Pacemakers
	Blood	Blood monitors
		Heart valves
		Vascular grafts
		Internal drug delivery catheter
		Permanent pacemaker electrodes

TABLE 23.19
Studies Often Conducted during the Safety Evaluation of Medical Devices

Cytotoxicity

- *In vitro* test that utilizes cell culture techniques
- Used primarily to evaluate the potential for local toxic effects
- Often used as an initial biological screen for new materials or changes to existing formulations (e.g., existing materials sterilized by a new method)

Sensitization

- Methods used most frequently utilize guinea pigs as the test organism
- Study duration of guinea pig assays is approximately 6 weeks
- Evaluates the sensitization component of the immunological response of the biological system to foreign materials
- Delayed hypersensitivity (type IV) allergic reaction is the specific biological reaction evaluated in this study
- Both natural and synthetic materials can produce sensitization reactions, and the potential morbidity that may be caused by sensitization of an individual to a device is significant
- All materials considered for use in medical devices should be evaluated for the potential to cause sensitization
- The local lymph node assay (LLNA) utilizing mice is becoming more widely accepted as a method (study duration is approximately 1 week)
- Using human volunteers to evaluate sensitization should be considered for some applications
- Study duration of human studies is approximately 6 weeks

Skin Irritation (Topical)

- Generally uses albino rabbits as the test model because of high potential for observable skin reaction in this model
- Most obviously used to evaluate potential for a topical product to produce skin irritation at the site of application
- Evaluation of treatment sites uses standardized scoring criteria to allow for a calculation of a "Primary Irritation Index" (combined severity of erythema and edema scores)
- For materials/devices intended to be in contact with abraded skin and/or open wounds, testing for skin irritation needs to be expanded to include animals in which the epidermis has been abraded using appropriate methods
- Study duration is approximately 3 days

Skin Irritation (Intracutaneous Reactivity)

- Uses albino rabbits as the test model because of high potential for observable skin reaction in this model
- Extracts injected into the surface layers of the skin (approximately between the epidermis and dermis)
- Evaluation of injection sites uses standardized scoring criteria similar to topical testing
- Has become a standard test to help evaluate the potential for many materials/devices, regardless of their intended final use, to produce a local reaction at the site of tissue contact
- Study duration is approximately 3 days

Acute Systemic Toxicity

- Utilizes mice injected with extracts by either the intravenous or ip route
- Designed to evaluate the potential for harmful effect(s) to occur distant from the site of tissue contact
- Evaluation for toxic effects generally follows standard toxicological evaluations including death, weight loss, and clinical signs
- Study duration is approximately 3 days

Implantation

- Used as a direct measure of the local irritation potential of materials/devices in direct contact with living tissue
- Implanted test article may be a small piece of a device component, a specific part of a final device, or an entire device
- Design often includes implantation of a standard control material into same animals (at distant sites) as the test article to serve as an irritant control
- Site of implantation is generally a large muscle of a laboratory animal (often an albino rabbit) due to ease of placement and subsequent gross and histopathological scoring of any tissue changes
- Care must be taken during test article preparation to avoid the inadvertent presence of any sharp edges/corners on the test article that may bias the results of the test by causing inflammation due to the shape of the test article
- Basic muscle implantation techniques do not absolutely require microscopic evaluation of the implantation site, but histology should be used as frequently as possible to maximize the potential to detect biologically significant reactions to the test article

(continued)

TABLE 23.19 (continued)**Studies Often Conducted during the Safety Evaluation of Medical Devices****Implantation**

- Attempts should be made to evaluate the tissue response macroscopically and microscopically without disturbing the test article location in the tissue since removal of the test article may also remove some inflammatory tissue from the site
- Specialized studies using implantation into the intended site of use of the device should be considered
- Method of inserting (implanting) the test article into the tissue has the potential to impact the results of the study
- Study duration can be 5 days to several months depending on proposed end use of the device

Hemocompatibility

- Evaluates the potential for a material/device to affect the formed cellular elements of the blood (cells/platelets) as well as to activate the coagulation and/or complement systems of the circulation
- Frequently conducted using *in vitro* assays in which the end point is red blood cell lysis, coagulation, etc.
- Assays generally use the blood from laboratory animals although this has been challenged due to possible differences in cell friability, and therefore, use of donated human blood may be considered
- Potential biological significance of hematological changes caused by materials/devices in contact with the blood suggests that *in vivo* testing should be considered when standard *in vitro* studies are inconclusive
- Study duration is generally very brief (less than 1 day)

Genotoxicity

- Assays evaluate the potential for test article to induce genetic changes including mutations and/or altered chromosomal structure
- *In vitro* studies including the Ames assay and chromosomal aberration are frequently used
- *In vivo* studies such as the mouse micronucleus assay may also be utilized
- Study duration varies with the specific assay

Subchronic/Chronic Systemic Toxicity

- These assays should only be conducted for devices used repeatedly (same device frequently on the same patient or different devices used frequently on the same patient)
- Studies are typically individually designed for specific devices
- Test system may be virtually any laboratory animal model and should be selected based upon the desired end point of the study
- Evaluation for toxic effects generally follows standard toxicological methods including death, weight loss, clinical signs, clinical pathology, hematology, and histopathology (of local tissue effects as well as possible target organs)
- These assays may be conducted by evaluating implanted materials (obviously, test and control articles must be placed into different animals for these assays) or by repeatedly dosing animals with freshly prepared extracts
- Study duration may be as short as 2 weeks or as long as several years depending upon the desired end point of the study

Carcinogenicity

- Rarely required for device materials
- Test system is generally mice or rats
- Evaluation for carcinogenicity end point follows standard toxicological methods and is dependent upon histopathology
- These assays may be conducted by evaluating implanted materials (obviously, test and control articles must be placed into different animals for these assays) or by repeatedly dosing animals with freshly prepared extracts
- Study duration (including significant time to complete histopathological evaluation) is at least 2 years and is often close to 3 years

CONSUMER PRODUCTS

OVERVIEW

This section is intended to provide general information on consumer products such as household and personal hygiene products for which a company might need to develop health and toxicity (safety) information. In the traditional sense and use of the term, standard household cleaning products are consumer products that are regulated under the Consumer Product Safety Act (CPSA) via the Consumer Product Safety Commission (CPSC). However, a household cleaning product that contains an antimicrobial agent and is labeled as a disinfectant is regulated under FIFRA, as administered by the EPA, because it contains a pesticide. Also consider the area of personal hygiene products, that is, “cosmetics” (Table 23.20). This general category includes such diverse products as shaving creams, shampoos, antibacterial liquid soaps, sunscreens, and athlete’s foot treatments, certainly all available directly to the consumer, that is, consumer products. However, the first two types of products are regulated under the FDCA as cosmetics, while the last three, because they contain an “active” ingredient, are regulated via the Over-the-Counter (OTC) Drug Monograph. And although both the FDCA and the OTC are administered by the FDA, the requirements for marketing these two types of products are quite different. And the previously mentioned product classes are not included in the special regulatory environments that exist for two other types of consumer products, art materials, and dietary supplements.

At least 10 major US federal regulations exist under which consumer products may be regulated. As previously illustrated, consumer products represent an extremely broad and diverse collection of substances, materials, and products, even if considered only within the context of the US market and regulatory environment, as will be the case in this section. For the purposes of this discussion, a consumer product is considered to be any product customarily produced or distributed for sale to or for consumption by the individual customer. Exceptions to this definition are foods and food additives, which are specially regulated by the FDCA/FDA; fuels, which are regulated primarily based on physiochemical hazard rather than toxicity; FIFRA/EPA-regulated pesticides available directly to the consumer; and prescription drugs, which are subjected by the FDA to the most comprehensive safety assessments in existence. OTC drug products are also a type of consumer product subject to toxicity testing.

General consumer product class definitions are presented in Table 23.21. The safety/toxicity testing of most consumer products, that is, household products and art supplies, is regulated under CPSA/CPSC. The majority of tests on consumer products are *in vitro* studies. Studies in animals are not frequently performed. In fact, testing of cosmetics on animals has been banned in the EU (European Union).

TABLE 23.20
Cosmetic Product Categories^a

Category	Product Types
Baby products	Shampoos
	Lotions
	Oils
	Powders
	Creams
	Other baby products
Bath preparations	Bath oils, tablets, salts
	Bubble baths
	Bath capsules
	Other bath preparations
Eye makeup preparations	Eyebrow pencil
	Eyeliner
	Eye shadow
	Eye lotion
	Eye makeup remover
	Mascara
	Other eye makeup preparations
Fragrance preparations	Colognes and toilet waters
	Perfumes
	Powders (dusting and talcum, excluding aftershave talc)
	Sachets
	Other fragrance preparations
Hair preparations (noncoloring)	Hair conditioners
	Hair sprays (aerosol fixatives)
	Hair straighteners
	Permanent waves
	Rinses (noncoloring)
	Shampoos (noncoloring)
	Tonics, dressings, and other hair grooming aids
	Wave sets
	Other hair preparations
Hair coloring preparations	Hair dyes and colors (all types requiring caution statement and patch test)
	Hair tints
	Hair rinses (coloring)
	Hair shampoos (coloring)
	Hair color sprays (aerosol)
	Hair lighteners with color
	Hair bleaches
	Other hair coloring preparations
Makeup preparations (not eye)	Blushers (all types)
	Face powders
	Foundations
	Leg and body paints
	Lipstick
	Makeup bases
	Rouges
	Makeup fixatives
	Other makeup preparations

(continued)

TABLE 23.20 (continued)
Cosmetic Product Categories^a

Category	Product Types
Manicuring preparations	Basecoats and undercoats
	Cuticle softeners
	Nail creams and lotions
	Nail extenders
	Nail polish and enamel
	Nail polish and enamel removers
	Other manicuring preparations
Oral hygiene products	Dentifrices (aerosol, liquid, pastes, and powders)
	Mouthwashes and breath fresheners (liquids and sprays)
	Other oral hygiene products
Personal cleanliness	Bath soaps and detergents
	Deodorants (underarm)
	Douches
	Feminine hygiene deodorants
Shaving preparations	Other personal cleanliness products
	Aftershave lotions
	Beard softeners
	Men's talcum
	Preshave lotions (all types)
	Shaving cream (aerosol, brushless, and lather)
	Shaving soap (cakes, sticks, etc.)
Skin care preparations (creams, lotions, powders, and sprays)	Other shaving preparations
	Cleansing (cold creams, cleansing lotions, liquids, and pads)
	Depilatories
	Face and neck (excluding shaving preparations)
	Body and hand (excluding shaving preparations)
	Foot powders and sprays
	Moisturizing
	Night
	Paste masks (mud packs)
	Skin fresheners
	Other skin preparations
Suntan preparations	Suntan gels, creams, and liquids
	Indoor tanning preparations
	Other suntan preparations

^a Taken from 21 CFR 720.4.

TABLE 23.21
General Consumer Product Class Definitions

Class	Definition
Household product	This may be viewed generally as a default category of products customarily manufactured or distributed for sale for consumption or use or customarily stored by individuals in or about the household that do not fall into other classes. Items such as toys, bunk beds, matches, charcoal briquettes, lighters, and electrical appliances are household products regulated under CPSA/CPSC; however, their regulation is based primarily on physical safety of the consumer and not on chemical toxicity hazard. For the purposes of this discussion, household products may be considered as chemicals or chemical mixtures to which consumers are exposed that do not fall into any of the other classes listed below.
Art material	"Any raw or processed material, or manufactured product, marketed or represented by the producer or repackager as intended for and suitable for [use by] artists or crafts people of any age who create, or recreate in a limited number, largely by hand, works which may or may not have a practical use, but in which aesthetic considerations are paramount" (16 CFR 1500.14)
Cosmetic	"A product which enhances appearance, aids in personal hygiene and does not affect the structure or function of the skin."
Disinfectant household product	Household products containing an active ingredient present to produce a specific biocidal (usually antimicrobial) effect that are marketed directly to the consumer. These materials are not intended for direct human application/exposure
Dietary supplement	Any product that is not a food or either a direct or indirect food additive that is intended for ingestion as a supplement to the diet
OTC drug product	Nonprescription drug or cosmetic-like products marketed directly to the consumer, deliberately ingested, or applied, containing an active ingredient present to produce a specific desired (usually biological) effect

REFERENCES

1. United States Environmental Protection Agency, Draft Report: Principles of neurotoxicity risk assessment, *Federal Register*, 58(148), 41556–41549, 1993.
2. Wexler, P., *Information Resources in Toxicology*, 2nd edn., Elsevier, New York, 1988, Chapter 10, pp. 245–249.
3. Merrill, R.A., Regulatory toxicology, in *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th ed., Amdur, M.D., Doull, J., and Klaassen, C.D., Eds., Pergamon Press, New York, 1991, Chapter 30, pp. 970–984.
4. U.S. Environmental Protection Agency, *Access EPA*, EPA 220B-92-014, 1992.
5. U.S. EPA, Office of Chemical Safety and Pollution Prevention, Harmonized Test Guidelines, EPA website, 2012, <http://www.epa.gov/ocspp/pubs/frs/home/guidelin.htm>.
6. U.S. EPA, Office of Chemical Safety and Pollution Prevention, Harmonized Test Guidelines, Series 870—Health Effects Test Guidelines, EPA website, 2012, http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series870.htm.
7. *Federal Register*, FR 32294, July 15, 1991.
8. *Code of Federal Regulations*, CFR Title 40.
9. *Federal Register*, 51, September 15, 1986.
10. *Federal Register*, 42 FR 45362, September 9, 1977.
11. *Federal Register*, 43 FR 11110, March 16, 1978.
12. Rubin, J.P. and Yaremchuk, M.J., Complications and toxicities of implantable biomaterials used in facial reconstructive and aesthetic surgery: A comprehensive review of the literature, *Plast. Reconstr. Surg.*, 100, 1336, 1997.
13. Gotman, I., Characteristics of metals used in implants, *J. Endourol.*, 11, 383, 1997.
14. Northup, S.J., Strategies for biological testing of biomaterials, *J. Biomater. Appl.*, 2, 132, 1987.
15. *U.S. Pharmacopeia 32/National Formulary 27*, April 30, 2010 edition, United States Pharmacopeial Convention, Inc., Rockville, MD, 2010, Chapter 88, p. 99.
16. Schuh, J., Medical device regulations and testing for toxicologic pathologists, *Toxicol. Pathol.* 36, 63, 2008.
17. Nair, P.D., Currently practiced sterilization methods—Some inadvertent consequences, *J. Biomater. Appl.*, 10, 121, 1995.
18. Schwindaman, D., Federal regulation of experimental animal use in the United States of America, *Rev. Sci. Technol.*, 13, 247, 1994.
19. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), ntp-server.niehs.nih.gov/htdocs/iccvam/REGUL.html.

Appendix A: LD₅₀ Values (Table A.1)

TABLE A.1

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Acetaldehyde						
Rabbit		300			1200	
Rat					640	1,900
Acetaminophen						
Rat			500			338
Acetanilide						
Mouse			820			
Rat			800			800
Acetone cyanohydrin						
Rabbit	17					
Acetylsalicylic acid						
Mouse			495			1,100
Rat			500			1,500
Guinea pig						1,200
Rabbit						1,800
Dog						3,000
Acetylcholine						
Mouse		20	>125		170	3,000
Rat		22			250	2,500
Rabbit		0.3				
Cat					10	
Aconitine						
Mouse		6.9				20
Albuterol sulfate						
Mouse						>2,000
Rat						>2,000
Alfentanil HCl						
Mouse		73				
Rat		45				
Guinea pig		75				
Dog		75				
Allobarbitol						
Rat					110	50
Alloxan						
Mouse		200	350			
Allopurinol						
Rat						4,500
Allylamine						
Mouse						57
Rat						106

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Allyl chloride						
Rat						700
α-Prodine						
Mouse		54	73		98	
Rat			22		23	90
Guinea pig		18				
Rabbit		18.5				
Alprazolam						
Rat						331–2,171
Altretamine						
Mouse						437
Rat						1,050
Amidephrine						
Mouse					1990	>6,000
Amiloride (base)						
Mouse						56
Rat						36–85
Aminocaproic						
Mouse		3 g/kg				12 g/kg
Rat		3.2 g/kg				16.4 g/kg
Aminogluthethimide						
Rat		156				1,800
Dog		>100				>100
Aminohippurate sodium						
Mouse, female		7.22 g/kg				
Aminophenazone						
Mouse		184			350	1,850
Rat		110				1,380
Rabbit						160
Dog						150
Aminophylline						
Mouse						540
Rabbit		150				
Aminopyrine						
Mouse		184			350	1,850
Rat			248			1,700
Amitriptyline						
Mouse		27	76		328	289
Rat		10	72		1290	530
Rabbit		9.9				446
Dog		10	72			200
Ammonium fluorosilicate						
Guinea pig						150
Amobarbital						
Rat		128	115			160
Rabbit		75				575
Dog		75				125
Amphetamine						
Mouse		25	120			22
Rat			125		160	60.5
Rabbit						85
Aniline						
Rat						440

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Anileridine (base)						
Mouse		25	53		100	128
Rat			45		163	175
<i>o</i> -Anisidine						
Mouse						1,300
Rat						1,400
Rabbit						2,900
<i>p</i> -Anisidine						
Mouse						1,400
Rat						2,000
Rabbit						870
Antazoline						
Rat					1.1 mmol/kg	
Antipyrine						
Mouse					1000	1,800
Rat						1,800
Apomorphine						
Dog		80				
Aprobarbital						
Rat					100	
Arsenic pentoxide						
Mouse						50–100
Rat						8
Arsenic trioxide						
Mouse						30–60
Rat						13–30
Asparaginase						
Mouse		500 K IU/kg				
Rabbit		22 K IU/kg				
Atracurium besylate						
Mouse, male		1.9				
Mouse, female		2				
Rat, male		1.3			283	
Atrazine						
Rat						1.2 g/kg
Atropine						
Mouse		90	250		900	400
Rat			280			750
Guinea pig			400			1,100
Auranofin						
Mouse						310
Rat						265
Azacyclonol						
Mouse		177	220		350	650
Azapetine						
Mouse		27	210	600	725	460
Rabbit		28				
Dog		50				
Azathioprine						
Mouse						2,500
Rat						400

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Aziridine						
Rat						15
Guinea pig	14					
Barbital						
Mouse			760			600
Barium chloride						
Mouse			500			
Rat					178	
Beclomethasone dipropionate						
Mouse						>1 g/kg
Rat						>1 g/kg
Bemegride						
Mouse		20	45		43	100
Rat		16.3	23.5		30.5	
Guinea pig		26.5				
Rabbit		25				
Benactyzine						
Mouse			100–130		250	350
Rat			100–130			
Benztropine						
Mouse		25			103	94
Rat					353	
Betaxolol						
Mouse						350–920
Rat						860–1,050
Bethanechol chloride						
Mouse						1,510
Bethanidine						
Mouse		12	150		260	520
Biperiden						
Mouse		56				545
Rat						750
Dog						340
Bisacodyl						
Mouse						17,500
Rat						4,320
Bis(2-chloroethyl)-ether						
Rat						75–150
Bitolterol mesylate						
Mouse						6,575
Rat						5,650
Bretylum						
Mouse		20	49		72	400
Bromoacetic acid						
Mouse						100
Bromomethane						
Rat						214
1-Bromopropane						
Rat						4,000
Buformin						
Mouse						380
Rat						320
Bulbocapnine						
Mouse					195	

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Bupivacaine HCl						
Mouse			6–8		38–54	
Bupropion HCl						
Mouse, male						544
Mouse, female						636
Rat, male						607
Rat, female						482
Buspirone						
Mouse						655
Rat						196
Dog						586
Monkey						356
Busulfan						
Mouse						120
1,4-Butynediol						
Rat						104
Guinea pig						130
Butyronitrile						
Mouse		50				
Rat						140–220
Cadmium chloride						
Rat						88
Cadmium cyanide						
Rat						16
Cadmium fluoride						
Guinea pig						150
Cadmium nitrate						
Mouse						100
Cadmium oxide						
Rat						72–296
Caffeine						
Mouse		100				1,200
Rat		105	245		250	200
Dog		175				
Calcium chloride						
Rat			500			4,000
Capreomycin sulfate						
Mouse					514	
Captan						
Rat						9,000–15,000
Carbachol						
Mouse		0.3			3	15
Rat		0.1			4	40
Carbamazepine						
Mouse			350			1,100–3,570
Rat						3,850–4,025
Rabbit						1,500–2,680
Guinea pig						920
Carbenicillin						
Mouse						3,600
Rat						2,000
Dog						>500
Rat		450				1,320
Rabbit		124				

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Carbromal						
Rabbit						500–700
Dog						450
Cefoxitin sodium						
Mouse, female		8 g/kg				
Rat			>10 g/kg			
Rabbit		>1 g/kg				
Ceftazidime						
Rabbit		>2 g/kg				
Cefuroxime sodium						
Mouse						>10 g/kg
Centchroman						
Mouse			400			
Chloral hydrate						
Mouse			890			
Rat					620	500
Chloralose						
Mouse			200			
Chlorambucil						
Mouse						123
Chlordiazepoxide						
Mouse		95	268		530	720
Rat		165			800	2,000
Rabbit		36				590
Dog						1,000
Chlorisondamine						
Mouse		24			401	
Rat		28				
Chloroacetic acid						
Mouse						165
Rat						76
Chloroacetonitrile						
Mouse						139
Rat						220
<i>p</i> -Chloroaniline						
Rat						310
1-Chloro-2,4-dinitrobenzene						
Rat						1,070
2-Chloroethanol						
Rat						58–95
Chloromethyl methyl ether						
Rat						817
<i>p</i> -Chloronitrobenzene						
Rat						810
Chloroprocaine HCl						
Mouse		97			950	
Chlorothiazide						
Mouse		1120				8,510
Rat			1,386			10,000
Dog		1000				>1,000
Chlorpheniramine						
Mouse						162
Chlorpromazine						
Mouse		26	92		300	319
Rat		29	74		542	493
Rabbit		235				
Dog		228				

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Chlorprothixene (2%)						
Mouse						350
Chlorprothixene (5% suspension)						
Mouse						220
Chlorprothixene (injectable)						
Mouse				>125		
Cimetidine						
Mouse		140				2,000–5,000
Rat		110				6,000
Clemastine						
Mouse						730
Rat						3,550
Dog						175
Clomethiazole						
Mouse		220				800
Clomipramine						
Mouse						630
Rat						1,450
Clonazepam						
Mouse						>2,000
Rat						>2,000
Clonidine HCl						
Mouse						206
Rat						465
Clotrimazole						
Mouse						700–1,000
Rat						700–1,000
Rabbit						700–1,000
Cocaethylene						
Mouse			60			
Cocaine						
Mouse		75–100	95			
Rat		17.5	70		250	
Rabbit		17				
Dog		22				
Codeine						
Mouse		68	130		183	395
Rat		55	102		332	542
Rabbit		60			32	
Colchicine						
Mouse		1.75	3.5		3.1	
Rat		1.7			4	
Cat		0.25				
Coniine						
Rabbit						56
Guinea pig						150
Cortisone acetate						
Mouse female			1,405			
<i>m</i> -Cresol						
Rat						2,020
<i>o</i> -Cresol						
Rat						1,350
<i>p</i> -Cresol						
Rat						1,800

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Crotonaldehyde						
Mouse						240
Cyanamide						
Rat						125
Cyclobarbitol						
Rat						205
Rabbit						450
Dog						250
Cyclobenzaprine HCl						
Mouse						338
Rat						425
Cycloserine						
Mouse						5,290
Cyclosporine						
Mouse		148				2,329
Rat		104				1,480
Rabbit		46				>1,000
Cyproheptadine						
Mouse		23	55		107	125
Rat			52			295
Dactinomycin						
Rat		0.46				
Decamethonium						
Mouse		0.75				
Rabbit		0.2				
Desipramine						
Mouse, male						290
Rat, female						320
Desmetryn						
Rat						1,390
Dexamethasone						
Mouse, female						6.5 g/kg
Dexamethasone sodium phosphate						
Mouse, female		794				
Dextroamphetamine						
Mouse		14.3	72.2		84	37
Rat					200	80
Dextromoramide						
Mouse						220
<i>o</i> -Dianisidine						
Rat						1,920
2,4-Diaminophenol						
Rat						240
Diazepam						
Mouse			220			970
Rat						1,200
Rabbit		8.8				
Dog						1,000
<i>N</i> -(2-chloroethyl)dibenzylamine						
Mouse			800			
Dibozane						
Mouse			260			
Rabbit		43				
3,4-Dichloroaniline						
Mouse						740
Rat						648

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Dichloroisoproterenol						
Mouse		48	132			
2,2'-dichloro-4,4'-methylenedianiline						
Mouse						880
1,1-Dichloro-1-nitroethane						
Rat						410
Dichlorphenamide						
Mouse						1,710
Rat						2,600
Dicyclomine HCl						
Mouse						625
Dicumarol						
Mouse		64	350			233
Rat		52				542
Guinea pig		59				
<i>N,N</i> -Diethylaniline						
Rat						782
Diethylcarbamazine						
Mouse						550
Rat						395
Diethylene glycol diacrylate						
Rat						770
Rabbit	180					
Diethylpropion HCl						
Mouse						600
Rat						250
Dog						225
Diflunisal						
Mouse, female						500
Rat, female						826
Digitoxin						
Mouse					22.2	32.7
Rat					16.4	23.8
Guinea pig						>100
Digoxin						
Guinea pig		0.355				
Dihydroergotamine						
Mouse		118				
Rat		110				
Rabbit		25				
Cat					68	
Diisopropyl fluorophosphate						
Mouse					3.7	36.8
Rat				1.8	3	6
Rabbit		0.34			1	9.8
Cat		1.6				
Dog		3.4			3	
Monkey		0.25				
Diltiazem HCl						
Mouse		60				415–740
Rat		38				560–810
Dog						>50
Dimethindene						
Rat		26.8				618.2
Guinea pig						888
Dog		45				

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
<i>N,N</i> -Dimethylaniline						
Rat						1,410
Dimethylnitrosamine						
Rat						26
Dimethylphenylpiperazinium						
Mouse			40	27.5		365
Rat						2,000
Dimethyl sulfate						
Rat						440
Dimethyl sulfoxide						
Mouse			14,700			
Dog						>10,000
2,4-Dinitrophenol						
Rat					25	30
Rabbit						200
Dog		30		20	22	25
2,4-Dinitrotoluene						
Rat						268
Diphenhydramine						
Mouse		31	84		127	164
Rat		42	82		475	500
Guinea pig			75			
Rabbit		10				
Dog		24				
Disopyramide phosphate						
Mouse						700
Rat						580
Dyclonine						
Mouse						90
Rat			31			176
Diphenylamine						
Rat						3,300
Doxapram HCl						
Mouse		75				
Rat		75				
Cat		40–80				
Dog		40–80				
Doxazosin						
Mouse						>1,000
Rat						>1,000
Doxepin						
Mouse		20				165
Rat		16				400
Doxycycline						
Mouse		175				1,600
Dropempine						
Mouse						420
Rat			125			370
Econazole						
Mouse						462
Rat						668
Guinea pig						272
Dog						>160

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Edrophonium						
Mouse		9	37		130	600
Rabbit		28.5				
Dog		15				
Enalaprilat						
Mouse, female		3740–5890				2,000
Rat						2,000
Ephedrine						
Mouse						1,550
Rat					650	
Epinephrine						
Mouse		0.5	4		1.47	50
Rat		0.98		3.5	5	
2,3-Epoxy-1-propanol						
Mouse						431
Rat						420
Rabbit	1980					
2,3-Epoxypropyl acrylate						
Rat						214
Rabbit	400					
Ergometrine						
Mouse		144				
Ergotamine						
Mouse		52				
Rat		62				
Rabbit		3.55				
Cat					11	
Ethacrynic acid						
Mouse						627
Ethacrynate sodium						
Mouse		175				
Ethanol						
Mouse		1,953	7,260		8,285	9,488
Rat			5,000			13,600
Guinea pig			5,560			
Rabbit						9,500
Ethinamate						
Rat						331
Dog						314
Ethosuximide						
Mouse						1,530
Rat						1,820
N-ethylaniline						
Mouse						500
Rat						290
Rabbit	4,700					
Ethyl biscoumacetate						
Mouse						880
Rat						880
Rabbit						1,100
Ethyl chloroacetate						
Rabbit	230					
Ethyl chloroformate						
Mouse						15
Rat						270
Rabbit	7,120					

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Ethylene dibromide						
Rat	300					
Rabbit	300					
Ethylene oxide						
Mouse						365
Rat						330
Ethyl methacrylate						
Rat						16 g/kg
Ethylmorphine						
Mouse					200	
Ethylnorepinephrine						
Mouse		117				
Etilefrine						
Mouse						1,600
Etretinate						
Rat			>4,000			>4,000
Mouse			>4,000			>4,000
Fentanyl citrate						
Rat		3				
Mouse		11.2				
Cat		1				
Dog		14				
Monkey		0.03				
Floxuridine						
Mouse		880				
Rat		670				
Rabbit		94				
Dog		157				
Fluoroacetic acid						
Mouse			10		16	8
Rat			0.4	5	2.5	2.5
Guinea pig			0.35			
Rabbit		0.25				
Cat		0.2				
Dog		0.06				
Monkey		4				
Fluorouracil						
Mouse		340				
Rat		165				
Rabbit		27				
Dog		32				
Flurazepam						
Mouse						870
Rat						1,230
Flurbiprofen						
Mouse			200			750
Rat			400			160
Fominoben						
Mouse		100				2,000
Rat		100				2,000
Fosinopril						
Rat						2,600
2-Furaldehyde						
Rat						127

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Furosemide						
Rat						4,600
Rabbit						800
Dog						2,000
Gadopentetate dimeglumine						
Mouse		5–12.5 mmol/kg				
Rat		10–15 mmol/kg				
Gallamine						
Mouse	4.3		9.6		17.4	425
Rat	5.5				25	
Rabbit	0.65			2.5	3	100
Dog	0.8					
Glucagon						
Mouse	300					
Glutethimide						
Mouse			350			
Guanethidine						
Mouse	22					
Rat	23				1,000	
Haloperidol						
Mouse	13				54	144
Rat	19					
Harmaline						
Mouse	120					
HC Blue No. 2						
Rat						1,250–5,000
Hemicholinium						
Mouse			0.064			
Rat			0.45			
Heparin						
Mouse	1780					
Hexachlorophene						
Rat	7.5		30			70
Hexamethonium						
Mouse	21		42		484	
Hexobarbital						
Mouse			340			468
Rat			280			468
Rabbit	80					
Hexocyclium						
Mouse	10.5		55		360	600
Histamine						
Guinea pig	0.18					
Homatropine						
Mouse			60		650	1,400
Rat			82		800	1,200
Guinea pig			120			1,000
Hydralazine						
Mouse			83			
Rat						180
Hydrazine						
Rat						60

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Hydrochlorothiazide						
Mouse		884	578		1470	3,080
Rat			234		1270	6,190
Rabbit	461					
Dog	250					
Hydrocortone						
Mouse					9	
Hydrocortisone						
Mouse, female			1,740			
Hydromorphone						
Mouse	88				84	
Hydroquinone						
Rat						700
Hydroxyzine						
Rat	45					1,000
Hyoscyamine sulfate						
Rat						375
Ibuprofen						
Mouse			320			800
Rat					1300	1,600
Imipramine						
Mouse	35		115		189	400
Rat	22		79		250	625
Rabbit	18					
Iodoacetic acid						
Mouse						83
Rat						60
Iodomethane						
Rat						76
Iohexol						
Mouse	24.2 g iodine/kg					
Rat	15 g iodine/kg					
Ipratropium bromide						
Mouse						1,001–2,010
Rat						1,667–4,000
Dog						400–1,300
Iproniazid						
Mouse	725		690	683	750	968
Rat						383
Rabbit	150					150
Isocarboxazid						
Mouse			110			173
Rat			199			280
Isoflurophate						
Mouse						37
Isoniazid						
Mouse	153		132	140	160	142
Rat	398				533	650
Rabbit	94				151	
Dog	50					
Isoprenaline						
Mouse						2,221
Isoproterenol						
Mouse	128		300			450
Guinea pig					0.32	270
Dog	50					

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Isosorbide dinitrate						
Rat						1,100
Ketamine						
Mouse		65	360.6			680
Rat		62				490
Labetalol						
Mouse		50–60				600
Rat		50–60				>2 g/kg
Lactulose						
Mouse						48.8 mL/kg
Rat						>30 mL/kg
Lauramine oxide (0.3%)						
Rat						>20 g/kg
Levallorphan						
Mouse			184			949
Rat			185			949
Levocarnitine						
Mouse						19.2 g/kg
Levorphanol						
Mouse		41.5	73		187	285
Rat					110	150
Rabbit		20				
Lidocaine HCl						
Mouse		31.5			400	457
Rat						459
Lignocaine						
Mouse		35	122			520
Lisinopril						
Mouse						>20 g/kg
Rat						>20 g/kg
Loperamide						
Rat		5.92				
Lysergide						
Mouse		54				46
Rat						16
Rabbit		3				
Malononitrile						
Mouse						18.6
Mebendazole						
Mouse						1,280
Rat						1,280
Guinea pig						1,280
Rabbit						1,280
Dog						>640
Cat						>640
Mebutamate						
Mouse			460			550
Rat			410			1,160
Mecamylamine (base)						
Mouse		21	39		93	92
Rat			54		145	171
Guinea pig			52		127	144
Mechlorethamine HCl						
Mouse		2				
Rat		1.6				

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Meclofenoxate						
Mouse		330				1,750
Meclozine						
Mouse						1,600
Menadiol sodium diphosphate						
Mouse		500				6,172
Rat		400				5,250
Mepenzolate						
Mouse		9.8				900
Rat		21.8				1,100
Meperidine						
Mouse		50	150		195	178
Rat		34	93		200	170
Rabbit		30				500
Mephenesin						
Mouse		186	471			990
Rat						625
Rabbit		125				
Mephenoxalone						
Mouse						3,820
Mephentermine						
Mouse			110			
Mephenytoin						
Mouse						560
Guinea pig			215			
Rabbit						430
Cat						190
Mepivacaine						
Mouse		23–35			280	
Meprobamate						
Mouse			710			980
Rat		350				1,600
Rabbit		260				
Mercaptopurine						
Mouse			250			
Mercury (II) bromide						
Mouse						35
Rat	100					1
Mercury (II) chloride						
Rat						1
Mercury (II) cyanide						
Mouse						33
Rat						26
Mercury (II) nitrate						
Rat						26
Mercury (II) oxide						
Mouse						22
Rat	315					18
Mescaline						
Mouse			500			
Rat			370			
Metaraminol						
Mouse						99
Rat						240

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Methacholine Cl						
Mouse						1,100
Rat						750
Methacrylonitrile						
Rat						0.25 mL/kg
Methadone						
Mouse		17	38		33	93.7
Rat		10	23		12	95
Guinea pig					54	
Monkey					15	
Methamphetamine						
Mouse		10	15			232
Methaqualone						
Rat		100				300
Metharbital						
Mouse		500				500
Methotrexate						
Mouse			4.5			
Methoxamine						
Mouse			92			
N-methylaniline						
Rabbit						280
Methylatropine						
Mouse		7	250			
2-Methylaziridine						
Rat						19
Methyl chloroformate						
Mouse	1750					
Rabbit	7120					
Rat						60
Methyldopa						
Mouse		1900	406			5,300
Rat			647			7,490
Rabbit						713
Methyldopate HCl						
Mouse		321				
Methylergonovine						
Mouse		85				187
Rat		23				93
Rabbit		2.6				4.5
Methyl isocyanate						
Mouse						120
Rat						69
Rabbit	0.22 mL/kg					90
1-Methyl-3-nitro-1-nitrosoguanidine						
Rat						90
Methyloxirane						
Rat						520–1,140
Methylpentynol						
Mouse						525
Rat						300–900
Guinea pig						534
Methylphenidate						
Mouse			450		470	680
Rat		48				367

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Metyrapone						
Rat						521
Miconazole nitrate						
Mouse						578
Rat						>640
Guinea pig						276
Dog						>160
Minoxidil						
Mouse		51	1,000–1,300			2,500
Rat		49				1,300–2,000
Morphine						
Mouse		275	500		500–700	745
Rat		237	500		266–572	905
Guinea pig					391	
Rabbit			500		600	
Muscarine						
Mouse		0.23				
Nalorphine						
Mouse		190	590	0.4	670	
Naloxone						
Mouse		150				565
Rat		109				
Naltrexone						
Mouse						1,100
Rat						1,450
Guinea pig						1,490
Naphazoline						
Mouse		170				
Rat				385		
Rabbit		0.8				
Naproxen						
Mouse						1,234
Rat						543
Hamster						4,110
Dog						>1,000
Neostigmine						
Mouse		0.36	0.62	0.31	0.8	14.4
Rat		0.16		0.42	0.37	
Rabbit				0.31		
Nialamide						
Mouse			742			1,000
Rat						1,700
Nicotine						
Mouse		7.1				3.3
Rat					33.5	>24
Rabbit		9.4				
Dog		5				
Nicotinic acid						
Rat						7,000
Nifedipine						
Mouse		16				490
Rat		4				1,020
Nikethamide						
Mouse			174			
Rat		191	300		470	
Rabbit			225			

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Nitrazepam						
Mouse						1,800
Rat						2,000
<i>m</i> -Nitroaniline						
Rat						535
<i>o</i> -Nitroaniline						
Rat						1,600
<i>p</i> -Nitroaniline						
Rat						750
Nitrofurazone						
Mouse						747
Rat					30	590
2-Nitro- <i>p</i> -anisidine						
Rat						14,100
2-Nitronaphthalene						
Rat						4,400
2-Nitropropane						
Rat						720
<i>o</i> -Nitrotoluene						
Rat						891
<i>p</i> -Nitrotoluene						
Mouse						1,231
Rat						2,144
5-Nitro- <i>o</i> -toluidine						
Rat						574
Nitroprusside						
Mouse		8.4				
Rat		11.2				
Rabbit		2.8				
Dog		5				
Nizatidine						
Mouse		232				
Rat		301				
Norephedrine						
Rabbit		75				
Norepinephrine						
Rat					29	132
Noscapine						
Rat						800
Obidoxime						
Mouse		100		200		2,240
Rat		100		200		
Rabbit		100		200		
Octreotide acetate						
Mouse		72				
Rat		18				
Orciprenaline						
Mouse						4,800
Osmium tetroxide						
Mouse						162
Ouabain						
Mouse			20			
Rat					97	
Guinea pig				0.26		
Cat		0.11				

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Oxazepam						
Mouse			>1,500			7,500
Oxilorphan						
Mouse		32			315	
Oxotremorine						
Mouse			5			
Papaverine						
Mouse		33.1	750			2,500
Rat			63		420	745
Paracetamol						
Rat						3,700
Paraldehyde						
Mouse						1,650
Guinea pig			1,230			
Rabbit		450				
Cat		450				
Dog		500				3,500
Paraquat (mmol/kg)						
Mouse			0.12			0.66
Rat			0.12			0.57–0.94
Guinea pig			0.018			0.19
Cat						0.22
Pargyline						
Mouse			370			680
Rat			142			300
Cat			200			
Dog						175
Monkey			150			
Pentachlorophenol						
Rat	96					
Pentazocine HCl						
Mouse						3,570
Pentobarbital						
Mouse		80	130	124	107	280
Rat			75			118
Guinea pig			50	70		
Rabbit		45				275
Cat						100
Pentolinium						
Mouse		29	36			512
Pentylene-tetrazol						
Mouse		51	92		101	162
Rat			70		100	
Perphenazine						
Mouse			70			
Phalloidine						
Mouse			1.9			
Phenacetin						
Mouse						1,030–2,000
Rat						1,650
Phenazone						
Rat						1,800
Phenelzine						
Mouse		157			150	156

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
<i>p</i> -Phenetidine						
Mouse						530
Rat						580
Pheniprazine						
Mouse		60.6	122		95	73
Rat		44.5			45.3	34.1
Phenobarbital						
Mouse			340		230	325
Rat			190		200	660
Rabbit		185				
Cat						175
Phenoxybenzamine						
Mouse						1,535
Rat						2,500
Guinea pig						500
Phentolamine						
Mouse						1,000
Rat		75			275	1,250
Phentolamine mesylate						
Mouse						1,000
Rat						1,250
Phenylbutazone						
Mouse		123	336			417.5
Rat		150	215			650–1,000
Rabbit						146
<i>m</i> -Phenylenediamine						
Rat						650
<i>p</i> -Phenylenediamine						
Rat						98
Phenylephedrine						
Mouse		21	1,000		70	120
Rat					92	350–1,120
Phenylhydrazine						
Mouse						175
Rat						188
Rabbit						80
Guinea pig						80
Phenyloxirane						
Rat						4,290
Rabbit	930–1060					
Phenytoin						
Mouse			200			490
Rat			280			
Rabbit		125				
Phloroglucinol						
Rat			3,180		4850	5,200
Physostigmine						
Mouse			1		0.54	3
Phytonadione (1%)						
Mouse		52 mL/kg				
Phytonadione						
Mouse			>25 g/kg			>25 g/kg
4-Picoline						
Rat						1,290
Rabbit	270					14.8

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Picrotoxin						
Mouse			7.2		7	14.8
Rat		3	6.5			
Pilocarpine						
Mouse			500			
Pimozide						
Mouse						>5,100
Rat						>5,100
Dog						40
Pipradrol						
Mouse			94		240	365
Rat		30			240	180
Rabbit		15				
Potassium bromate						
Rat			50–200			200–400
Potassium cyanide						
Mouse					6	16
Potassium fluorosilicate						
Guinea pig						500
Pralidoxime						
Mouse		90	155	180		4,100
Rat		96		150		
Guinea pig				168		
Rabbit		95				
Praziquantel						
Mouse						2,500
Rat						2,500
Prednisolone phosphate disodium						
Mouse, female			1,190			
Prenylamine						
Mouse						200
Rat						1,000
Primidone						
Mouse						600–800
Rat						1,500–2,000
Proadifen						
Mouse		60	117.5			538
Rat			163			2,140
Probenecid						
Mouse		458	230		1,156	1,666
Rat			394		611	1,604
Rabbit		304				
Dog		270				
Probucol						
Mouse						>5 g/kg
Rat						>5 g/kg
Procaine						
Mouse		45	230	630	800	500
Rat		50	250	1600	2100	
Guinea pig		51				
Rabbit		57				
Dog		62.4				
Prochlorperazine						
Mouse		92	125		350	750
Rat						1,800

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Prodilidine						
Mouse		91			194	318
Rat		74			188	253
Promazine						
Mouse		38		113		216–485
Rat		17–29		233		343–650
Rabbit		21				125
Promethazine						
Mouse		28–75	150	216	750	375–575
Rat		20–45		250	225	480
Guinea pig		42.5				
Rabbit		19				
Prontalol						
Mouse		50	124			900
Rat		50				900
Propanidid						
Mouse		90				
Rat		80				
Dog		80				
Rabbit		75				
Propantheline						
Rat					298	370
Propargyl alcohol						
Mouse						50
Rat						55
Guinea pig						60
Propiverine						
Mouse		113				490
Rat		29				2,100
Rabbit		13				620
Propofol						
Mouse		53				
Propoxyphene HCl						
Mouse					204	282
Rat					131	230
Propoxyphene napsylate						
Mouse						915
Rat						647
Rabbit						>183
Dog						>183
Propranolol						
Mouse		27	114			380
Rat						533
Propyl chloroformate						
Mouse						650
Protoveratrine						
Mouse		0.05	0.4			
Rat					0.6	5
Rabbit		0.05			0.11	
Cat					0.5	
Protriptyline						
Mouse		37			192	269
Pseudoephedrine						
Mouse						726
Rat			202			2,206
Rabbit						1,177

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Psilocin						
Mouse		74				
Rat		75				
Psilocybin						
Mouse		285				
Rat		280				
Pyrethrin						
Rat						1,500
Pyrilamine						
Mouse		30	102		150	235
Rat					150	
Guinea pig		24.4			70	
Quinidine						
Mouse		69	190	200		594
Rat		23.1				1,000
Cat		21.6				
Ramipril						
Mouse						10–11 g/kg
Rat						10–11 g/kg
Ranitidine HCl						
Mouse		77				
Rat		83				
Reserpine						
Mouse			70			390–500
Rat		18				
Resorcinol						
Rat						370
Guinea pig						370
Rifampin						
Mouse						885
Rat						1,720
Rabbit						2,120
Ritodrine						
Mouse						540
Rabbit		64				
Rotenone						
Rat						100–300
Salicylamide						
Mouse		313				1,400
Rat						1,200
Rabbit			600			3,000
Scopolamine						
Mouse			153.5		590	
Secobarbital						
Cat						50
Selenium						
Rat						6,700
Selenium disulfide						
Rat						138
Selenium monosulfide						
Mouse						370
Selenium tetrachloride						
Guinea pig					19	
Semicarbazide						
Mouse		125.6	23.3		125.5	176

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Serotonin						
Mouse		160	868	750		
Rat		30	117			
Sodium bromide						
Mouse					5,020	7,000
Rat						3,500
Sodium fluoride						
Rat						200
Sodium tetradecyl sulfate						
Mouse		90				
Rat		72–128				
Solanine						
Rat			75			590
Rabbit			20–23			
Sheep						225
Sotalol						
Mouse			670			2,600
Rat			680			3,450
Rabbit						1,000
Dog			330			
<i>g</i> -Strophanthin						
Guinea pig				0.26		
Stearyl Heptanoate						
Rat						>5 g/kg
Strychnine						
Mouse			0.98		0.85	
Rat			0.09–1.4		1.2	16.2
Succinylcholine						
Mouse		0.75	4			125
Rabbit		1				
Sulfacetamide						
Mouse						16.5 g/kg
Sulfadimethoxine						
Rat						>4,000
Rabbit						>2,000
Sulfafurazole						
Rat						>10,000
Rabbit						>2,000
Sulfaguanidine						
Mouse						15,000
Sulfamethoxazole						
Mouse						3,200
Sufentanil citrate						
Mouse		17				
Rat		10.5				
Guinea pig		12.5				
Dog		10.1–19.5				
Sulfamethoxazole						
Mouse						2,300
Rat						3,000
Rabbit						>2,000
Sulfanilamide						
Mouse						3,700–4,300
Rat						3,900–10,000
Dog						2,000

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Sulfasalazine						
Mouse						>12 g/kg
Sulfisoxazole						
Mouse						5,700
Rat						>10,000
Rabbit						>2,000
Syrosingopine						
Rat		50				
Talinolol						
Mouse						600–1,450
Rat						1,180–2,580
Terbutaline						
Mouse						3,000
Rat						18,000
Terconazole						
Rat, male						1,741
Rat, female						849
Dog, male						1,280
Dog, female						640
Terfenadine						
Mouse						>5,000
Rat						>5,000
Rat newborn						438
Tetrabenazine						
Mouse		150			400	
Tetracaine						
Mouse		6.6				
Guinea pig		15.6				
Dog		4.3				
1,1,2,2-Tetrabromoethane						
Mouse						269
Rat	5,250					1,100
Rabbit						400
Guinea pig						400
1,1,2,2-Tetrachloroethane						
Rat						800
2,3,4,5-Tetrachlorophenol						
Mouse						400
Rat						140
Guinea pig						250
2,3,4,6-Tetrachlorophenol						
Mouse						109
Rabbit	250					
Tetraethylammonium						
Mouse		29	56			655
Rat		63	115			
Rabbit		72				
Dog		55				
Tetrahydrocannabinol						
Mouse			510			
Tetryzoline						
Mouse		39				
Thebaine						
Rat						13.9

TABLE A.1 (continued)**LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)**

	Dermal	IV	IP	IM	SC	PO
Theophylline						
Mouse						350
Thioglycolic acid						
Rat						114
Rabbit	848					
Thioguanine						
Rat, male						823
Rat, female						740
Thiram						
Rat						865
Rabbit						210
Thiopental						
Mouse		112	200			350
Rat		67.5	120			
Guinea pig		55	57.5			
Rabbit		40				600
Dog		55				150
Timolol maleate						
Mouse, female						1,190
Rat, female						900
Tocainide HCl						
Mouse						800
Rat						1,000
Guinea pig						230
Tolazoline						
Mouse			500			
<i>o</i> -Tolidine						
Rat						404
Toluene-2,4-diisocyanate						
Rat						5,800
<i>o</i> -Toluidine						
Rat						670
Rabbit	3250					
P-Toluidine						
Mouse						794
Rat						656
Rabbit	890					
Toxaphene						
Rat						40–120
Tranylcypromine						
Mouse		37				38
Trazodone						
Mouse		96				610
Rat						486
Rabbit						560
Triamterene						
Mouse						300–380
Triazolam						
Mouse						>1,000
Rat						>5,000
Trichloroacetonitrile						
Rat						250
Rabbit	900					
Trichloronitromethane						
Rat						250

(continued)

TABLE A.1 (continued)

LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
Trichlorotrinitrobenzene						
Rat						30 g/kg
Trichothecene						
Rat		0.75				
Guinea pig		1.3				
Trifluoperidol						
Mouse						988
Rat						113
Trifluoperazine						
Mouse		36				442
Rat						740
Dog		60				
Trimethadione						
Mouse		2000	1,800			2,200
Rat					2200	
Rabbit			1,500	1,500		
Trimipramine						
Mouse						250
Tripelennamine						
Mouse		17	70		75	210
Rat		13			225	570
Guinea pig					30.2	155
Triphenyltin acetate						
Rat						140
Rabbit						30
Triphenyltin hydroxide						
Mouse						245
Rat						46
Tubocurarine						
Mouse			0.14			
Rat			0.25			
Rabbit		0.35				
Ursodiol						
Mouse						>7,500
Rat						>5,000
Vancomycin HCl						
Mouse		400				
Rat		319				
Veratridine						
Mouse		0.42	1.35			
Rat			3.5			
Vidarabine						
Mouse						>5,020
Rat						>5,020
Vinyl chloride						
Rat						500
Vinylcyclohexene diepoxide						
Rat						2,130
Rabbit	620					
Warfarin						
Mouse		165				374
Rat		186				323
Guinea pig						182
Rabbit		150				800
Dog		250				250

TABLE A.1 (continued)
LD₅₀ Values of Common Xenobiotics (mg/kg Unless Stated Otherwise)

	Dermal	IV	IP	IM	SC	PO
2,4-Xylenol						
Mouse	1040					
Rat						3,200
2,5-Xylenol						
Mouse						383
2,6-Xylenol						
Mouse	920					980
Rat						296
Zinc phosphide						
Mouse						40
Rat						2.7–40.5
Rabbit	2000–5000					40
Cat						250
Zoxazolamine						
Mouse			376			825
Rat			102			376
Dog		117				

Sources: Borchard, R.E. et al., Eds., *Drug Dosage in Laboratory Animals: A Handbook*, CRC Press, Boca Raton, FL, 1992; *Chemical Safety Data Sheets*, Vols. 4a and 4b, Royal Society of Chemistry, Indispensable Publications Ltd., Northamptonshire, U.K., 1991; *Physician Desk Reference*, 47th edn., Medical Economics Data, Montvale, NJ, 1993; Müller, K.R., Ed., *Toxicological Analysis*, Ullstein Mosby GmbH & Co., KG, Berlin, Germany, 1992.

REFERENCES

Borchard, R.E. et al., Eds., *Drug Dosage in Laboratory Animals: A Handbook*, CRC Press, Boca Raton, FL, 1992.
Chemical Safety Data Sheets, Vols. 4a and 4b, Royal Society of Chemistry, Indispensable Publications Ltd., Northamptonshire, U.K., 1991.

Müller, K.R., Ed., *Toxicological Analysis*, Ullstein Mosby GmbH & Co., KG, Berlin, Germany, 1992.
Physician Desk Reference, 47th edn., Medical Economics Data, Montvale, NJ, 1993.

Appendix B: Tables of Comparative Anatomical, Physiological, and Biochemical Data (Tables B.1 through B.14)

TABLE B.1

Comparison of Physiological Parameters for Different Body Organs

Organ	Weight (kg)	Percent of Body Volume	Percent Water	Blood Flow (mL/min)	Plasma Flow (mL/min)	Blood Flow (mL/kg)	Blood Flow Fraction
Adrenal glands		0.03		25	15		
Blood	5.4	7	83	5000			
Bone	10	16	22	250	150		
Brain	1.5	2	75	700	420	780	
Fat	10	10	10	200	120		0.05
Heart	0.3	0.5	79	200	120	250	
Kidneys	0.3	0.4	83	1100	660	1200	
Liver	1.5	2.3	68	1350	810	1500	0.25
Portal				1050	630		
Arterial				300	180		
Lungs	1.0	0.7	79	5000	3000		
Muscle	30	42	76	750	450	900	0.19
Skin	5	18	72	300	180	250	
Thyroid gland	0.03	0.03		50	30		
Total body		100	60	5000	3000		

Source: Modified from Illing, *Xenobiotic Metabolism and Disposition: The Design of Studies on Novel Compounds*, CRC Press, Boca Raton, FL, 1989.

Note: Data are for hypothetical 70 kg human.

TABLE B.2
Comparison of the Blood Flow/Perfusion and Oxygen
Consumption of Liver, Lung, Intestine, and Kidney of the Rat
***In Vivo* and in Organ Perfusion^a**

Parameter (Unit)	Liver	Lung	Intestine	Kidney
<i>In vivo</i>				
Blood flow (mL/min)	13–20	55–70	5–8	4–6
Blood pressure S/D (torr)	150/100	25/10	150/100	150/100
pO ₂ (arterial) (torr)	95	40	95	95
pO ₂ (venous) (torr)	40	100	50	70
O ₂ consumption (μL/min)	500–800	From air	40–160	100–200
<i>In perfusion</i>				
Perfusion flow (mL/min)	30–50	50	6	20–35
Perfusion pressure (torr)	100–120	10–20	100–120	100–120
pO ₂ (arterial) (torr)	600	600	400	600
pO ₂ (venous) (torr)	200	?	180	400
Max. O ₂ supply ^b (μL/min)	380–630	?	120 ^c	120–220

Source: *Toxicology: Principles and Applications*, CRC Press, Boca Raton, FL, 1996. With permission.

Note: S = systolic; D = diastolic.

^a These values are indications of the most common values measured for the various organs in a rat of 250–300 g. The figures provided for the kidney apply to a single kidney. The values measured in organ perfusions may differ greatly, depending on the setup, method of gassing, etc.

^b Calculated from pO₂ (arterial), pO₂ (venous), and perfusion flow.

^c With 20% FC-43 emulsion in KRB; other figures apply to KRB buffer without erythrocytes or oxygen carrier (KRB = Krebs–Ringer buffer).

TABLE B.3
Comparison of Respiratory Volume and Alveolar Ventilation
in Relation to Animal Weight

Species	Weight (g)	Respiratory Volume per Minute (cm ³)	Respiratory Volume per Minute (cm ³)/ Weight(g)	Alveolar Ventilation (L/h)/Weight (g)
Mouse	19.8	24.5	1.24	0.067
Cotton rat	76.8	39.6	0.52	
Hamster	91.6	60.9	0.67	
White rat	112.8	72.9	0.65	0.070
Guinea pig	466.0	155.6	0.33	
Rabbit	2,069.0	800.0	0.39	
Monkey	2,682.0	863.5	0.32	
Human	68,500.0	8,732.0	0.13	0.005

Source: Modified from Calabrese, E.J., Ed., *Principles of Animal Extrapolation*, Lewis Publishers, Chelsea, MI, 1991.

TABLE B.4
Comparison of Dosage by Weight and Surface Area

Species	Weight (g)	Dosage (mg/kg)	Dose (mg/Animal)	Surface Area (cm ²)	Dosage (mg/cm ²)
Mouse	20	100	2	46	0.043
Rat	200	100	20	325	0.061
Guinea pig	400	100	40	565	0.071
Rabbit	1,500	100	150	1,270	0.118
Cat	2,000	100	200	1,380	0.145
Monkey	4,000	100	400	2,980	0.134
Dog	12,000	100	1,200	5,770	0.207
Human	70,000	100	7,000	18,000	0.388

Source: Amdur, M.O. et al., Eds, *Casarett and Doull's Toxicology*, 4th edn., Pergamon Press, New York, 1991. With permission.

TABLE B.5
Relationship between Body Weight and Body Surface Area in a Number of Vertebrates

Species	Weight (g)	Surface Area (cm ²)
Mouse	20	46
Rat	200	325
Guinea pig	400	565
Rabbit	1,500	1,270
Cat	2,000	1,380
Monkey	4,000	2,980
Dog	12,000	5,770
Man	70,000	18,000

Source: From *Toxicology: Principles and Applications*, CRC Press, Boca Raton, FL, 1996. With permission.

TABLE B.6
Comparison of Bile Flow and Hepatic Blood Flow Rates in Various Species

Species	Liver Weight as Percentage of Total Body Weight	Hepatic Blood Flow Rate (mL Blood/100 g Liver/min)	Bile Flow Rate (mL Bile/kg Body Weight/Day)
Rat	3.36	79	28.6–47.1
Guinea pig	3.86	—	228
Rabbit	3.2	74	118
Cat	3.59	35–48	14
Dog	2.94	82	12
Hen	1.53	—	14
Sheep	2.97	—	12
Monkey	2.09	—	28

Source: From Calabrese, E.J., Ed., *Principles of Animal Extrapolation*, Lewis Publisher, Chelsea, MI, 1991. With permission.

TABLE B.7**Comparison of the pH Value of Contents of Different Parts of the Alimentary Tract in Various Species**

Species (No. Examined)	pH Value (Median for Different Animals) of Contents of								
	Stomach ^a		Small Intestine Portion				Cecum	Colon	Feces ^a
	A	P	1	3	5	7			
Monkey (3)	4.8	2.8	5.6	5.8	6.0	6.0	5.0	5.1	5.5
Dog (3)	5.5	3.4	6.2	6.2	6.6	7.5	6.4	6.5	6.2
Cat (6)	5.0	4.2	6.2	6.7	7.0	7.6	6.0	6.2	7.0
Ox (3)	6.0	2.4	6.7	7.0	7.3	7.9	7.0	7.4	7.5
Sheep (3)	6.4	3.0	5.7	6.6	7.7	8.0	7.3	7.8	8.0
Horse (3)	5.4	3.3	6.7	7.0	7.3	7.9	7.0	7.4	7.5
Pig (20)	4.3	2.2	6.0	6.2	6.9	7.5	6.3	6.8	7.1
Rabbit (11)	1.9	1.9	6.0	6.8	7.5	8.0	6.6	7.2	7.2
Guinea Pig (6)	4.5	4.1	7.6	7.7	8.1	8.2	7.0	6.7	6.7
Rat (7)	5.0	3.8	6.5	6.7	6.8	7.1	6.8	6.6	6.9
Mouse (3)	4.5	3.1	—	—	—	—	—	—	—
Hamster (3)	6.9	2.9	6.1	6.6	6.8	7.1	7.1	—	—
Gerbil (3)	5.5	3.8	6.7	7.0	7.8	8.2	7.0	7.4	7.5
Fowl (6)	4.9	4.2	5.8	6.2	7.0	7.8	7.0	—	7.6
Duck (3)	5.0	4.5	6.1	6.5	7.4	8.0	—	—	7.3

Source: From Calabrese, E.J., Ed., *Principles of Animal Extrapolation*, Lewis Publisher, Chelsea, MI, 1991. With permission.

^a A = anterior portion of stomach; P = posterior portion; feces = contents of posterior rectum.

TABLE B.8**Comparison of Enzyme Activity in the Small Intestine of Several Species**

Species	Enzymatic Activity in the Small Intestine as a Percentage of That in the Liver					
	Ethylmorphine N-Demethylase	Biphenyl Hydroxylase	Aniline Hydroxylase	AHH	Cytochrome-c Reductase	Cytochrome P-450
Rabbit	18.6	14.1	20.4	30.0	75.7	34.6
Guinea pig	23.3	16.4	19.8	37.4	78.7	12.4
Cat	ND	9.3	ND	4.6	42.0	ND
Mouse	ND	9.0	ND	6.0	79.6	4.0
Hamster	ND	6.8	ND	5.7	60.7	13.0

Source: From *Toxicology: Principles and Applications*, CRC Press, Boca Raton, FL, 1996. With permission.

Note: ND = not detectable.

TABLE B.9**Comparison of pH Values in Some Human Body Compartments**

Blood	7.35–7.45
Oral cavity	6.2–7.2
Stomach (at rest)	1.0–3.0
Duodenum	4.8–8.2
Jejunum	6.3–7.3
Ileum	7.6
Colon	7.8–8.0
Rectum	7.8
Cerebral fluid	7.3–7.4
Vagina	3.4–4.2
Urine	4.8–7.5
Sweat	4.0–6.8
Milk	6.6–7.0

Source: From Niesink, R.J.M., de Vries, J. and Hollinger, M.A., *Toxicology: Principles and Applications*, CRC Press, Boca Raton, FL, 1996. With permission.

TABLE B.10
Comparison of the Size^a of the Absorptive Surface
of the Various Parts of the Gastrointestinal Tract

Oral cavity	0.02
Stomach	0.1–0.2
Small intestine	100
Large intestine	0.5–1.0
Rectum	0.04–0.07

Source: From *Toxicology: Principles and Applications*, CRC Press, Boca Raton, FL, 1996. With permission.

^a Relative size (e.g., surface area of the small intestine is ~500–1000 times greater than the surface area of the stomach).

TABLE B.11
Comparison of Physiological Characteristics of Experimental Animals and Humans

Species	Body wt. (kg)	Surface Area (m ²)	Cardiac Function							Arterial Blood Pressure (mm Hg)	
			Energy Metabolism ^a		Heart wt. (g/100 g)	Heart Rate (beats/min)	Stroke Vol (mL/beat)	Cardiac Output (L/min)	Cardiac Index (L/m ² /m)	Systolic	Diastolic
			(cal/kg/day)	(cal/m ² /day)							
Rat	0.1–0.5	0.03–0.06	120–140 (B)	760–905 (B)	0.24–0.58	250–400	1.3–2.0	0.015–0.079	1.6	88–184	58–145
Rabbit	1–4	0.23	47	810	0.19–0.36	123–330	1.3–3.8	0.25–0.75	1.7	95–130	60–90
Monkey	2–4	0.31	49 (B)	675	0.34–0.39	165–240	8.8	1.06	—	137–188	112–152
Dog	5–31	0.39–0.78	34–39 (B)	770–800 (B)	0.65–0.96	72–130	14–22	0.65–1.57	2.9	95–136	43–66
Man	54–94	1.65–1.83	23–26 (B)	790–910 (B)	0.45–0.65	41–108	62.8	5.6	3.3	92–150	53–90
Pig	100–250	2.9–3.2	14–17 (B)	1100–1360 (B)	0.25–0.40	55–86	39–43	5.4	4.8	144–185	98–120
Ox	500–800	4.2–8.0	15 (B)	1635 (B)	0.31–0.53	40–58	244	146	—	121–166	80–120
Horse	650–800	5.8–8.0	25 (R)	2710–2770 (R)	0.39–0.94	23–70	852	188	4.4	86–104	43–86

Source: Mitruka, B.M. and Rawnsley, H.M., *Clinical, Biochemical and Hematological Reference Values in Normal Experimental Animals*, Masson Publishing, New York, 1977. With permission.

^a B = basal; R = resting.

TABLE B.12**Comparison of Certain Physiological Values of Experimental Animals and Humans**

Species	Body Temperature (°C)	Whole Blood Volume (mL/kg body wt)	Plasma Volume (mL/kg body wt)	Plasma pH	Plasma CO ₂ Content (mM/L)	CO ₂ Pressure (mm Hg)
Mouse	36.5 ± 0.70	74.5 ± 17.0	48.8 ± 17.0	7.40 ± 0.06	22.5 ± 4.50	40.0 ± 5.40
Rat	37.3 ± 1.40	58.0 ± 14.0	31.3 ± 12.0	7.35 ± 0.09	24.0 ± 4.70	42.0 ± 5.70
Hamster	36.0 ± 0.50	72.0 ± 15.0	45.5 ± 7.50	7.39 ± 0.08	37.3 ± 2.50	59.0 ± 5.00
Guinea pig	37.9 ± 0.95	74.0 ± 7.00	38.8 ± 4.50	7.35 ± 0.09	22.0 ± 6.60	40.0 ± 9.80
Rabbit	38.8 ± 0.65	69.4 ± 12.0	43.5 ± 9.10	7.32 ± 0.03	22.8 ± 8.60	40.0 ± 11.5
Chicken	41.4 ± 0.25	95.5 ± 24.0	65.6 ± 12.5	7.52 ± 0.04	23.0 ± 2.50	26.0 ± 4.50
Cat	38.6 ± 0.70	84.6 ± 14.5	47.7 ± 12.0	7.43 ± 0.03	20.4 ± 3.50	36.0 ± 4.60
Dog	38.9 ± 0.65	92.6 ± 29.5	53.8 ± 20.1	7.42 ± 0.04	21.4 ± 3.90	38.0 ± 5.50
Monkey	38.8 ± 0.80	75.0 ± 14.0	44.7 ± 13.0	7.46 ± 0.06	29.3 ± 3.8	44.0 ± 4.8
Pig	39.3 ± 0.30	69.4 ± 11.5	41.9 ± 8.90	7.40 ± 0.08	30.2 ± 2.5	43.0 ± 5.60
Goat	39.5 ± 0.60	71.0 ± 14.0	55.5 ± 13.0	7.41 ± 0.09	25.2 ± 2.8	50.0 ± 9.40
Sheep	38.8 ± 0.80	58.0 ± 8.50	41.9 ± 12.0	7.48 ± 0.06	26.2 ± 5.00	38.0 ± 8.50
Cattle	38.6 ± 0.30	57.4 ± 5.00	38.8 ± 2.50	7.38 ± 0.05	31.0 ± 3.0	48.0 ± 4.80
Horse	37.8 ± 0.25	72.0 ± 15.0	51.5 ± 12.0	7.42 ± 0.03	28.0 ± 4.00	47.0 ± 8.50
Man	36.9 ± 0.35	77.8 ± 15.0	47.9 ± 8.70	7.39 ± 0.06	27.0 ± 2.00	42.0 ± 5.00

Source: Mitruka, B.M. and Rawnsley, H.M., *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals*, Masson Publishing, New York, 1977. With permission.

TABLE B.13**Comparison of Biochemical Components in Urine of Normal Experimental Animals and Humans**

Component (mg/kg body wt/day) or Property	Rat	Rabbit	Cat	Dog	Goat	Sheep
Volume (mL/kg body wt/day)	150–350	20.0–350	10.0–30.0	20.0–167	7.0–40.0	10.0–40.0
Specific gravity	1.040–1.076	1.003–1.036	1.020–1.045	1.015–1.050	1.015–1.062	1.015–1.045
pH	7.30–8.50	7.60–8.80	6.00–7.00	6.00–7.00	7.5–8.80	7.50–8.80
Calcium	3.00–9.00	12.1–19.0	0.20–0.45	1.00–3.00	1.00–3.40	1.00–3.00
Chloride	50.0–75.0	190–300	89.0–130	5.00–15.0	186–376	—
Creatinine	24.0–40.0	20.0–80.0	12.0–30.0	15.0–80.0	10.0–22.0	5.80–14.5
Magnesium	0.20–1.90	0.65–4.20	1.50–3.20	1.70–3.00	0.15–1.80	0.10–1.50
Phosphorous, inorganic	20.0–40.0	10.0–60.0	39.0–62.0	20.0–50.0	0.5–1.6	0.10–0.50
Potassium	50.0–60.0	40.0–55.0	55.0–120	40.0–100	250–360	300–420
Protein, total	1.20–6.20	0.74–1.86	3.10–6.82	1.55–4.96	0.74–2.48	0.74–2.17
Sodium	90.4–110.0	50.0–70.0	—	2.00–189	140–347	0.80–2.00
Urea nitrogen (g/kg/day)	1.00–1.60	1.20–1.50	0.80–4.00	0.30–0.50	0.14–0.47	0.11–0.17
Uric acid	8.00–12.0	4.00–6.00	0.20–13.0	3.1–6.0	2.00–5.00	2.00–4.00
	Swine	Cattle	Horse	Monkey	Man	
Volume (mL/kg body wt/day)	5.00–30.0	17.0–45.0	3.0–18.0	70.0–80.0	8.60–28.6	
Specific gravity	1.010–1.050	1.025–1.045	1.020–1.050	1.015–1.065	1.002–1.040	
pH	6.25–7.55	7.60–8.40	7.80–8.30	5.50–7.40	4.80–7.80	
Calcium	—	0.10–3.60	—	10.0–20.0	0.60–8.30	
Chloride	—	10.0–140	81.0–120	80.0–120	40.0–180	
Creatinine	20.0–90.0	15.0–30.0	—	20.0–60.0	15.0–30.0	
Magnesium	—	2.00–7.00	—	3.20–7.10	0.42–2.40	
Phosphorous, inorganic	—	0.01–6.20	0.05–2.00	9.00–20.6	10.0–15.0	
Potassium	—	240–320	—	160–245	16.0–56.0	
Protein, total	0.33–1.49	0.25–2.99	0.62–0.99	0.87–2.48	0.81–1.86	
Sodium	—	2.00–40.0	—	—	25.0–94.0	
Urea nitrogen (g/kg/day)	0.28–0.58	0.05–0.06	0.20–0.80	0.20–0.70	0.20–0.50	
Uric acid	1.00–2.00	1.00–4.00	1.00–2.00	1.00–2.00	0.80–3.00	

Source: Mitruka, B.M. and Rawnsley, H.M., *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals*, Masson Publishing, New York, 1977. With permission.

TABLE B.14**Comparison of Some Biochemical/Physiological/Morphological Differences of Potential Toxicological Significance between Rats and Humans**

	Rat	Human
A. Skin characteristics		
1. Stratum corneum		Much thicker than rat
2. Dermal vasculature		Much thicker than rat
3. Sweat glands	Missing from general body surface; eccrine sweat glands located in foot pads to moisten frictional surface	Numerous coiled tubular sweat glands (100–600/m ²)
4. Hair follicles	Densely haired with up to 4000 hairs/cm ²	Much fewer hairs, with 40–70 hairs/cm ² on skin of the trunk and limbs
5. Dermal absorption based on the aforementioned characteristics	Considerably more efficient absorber than humans for a wide variety of organic compounds	
B. Respiratory parameters		
1. Histamine content (µg/g)	15.8	27.7
2. Exogenous histamine catabolism (%)	44.2	29.2
3. Histamine release (%) µg/g		
Compound 48/80	17.1	43.2
Cotton dust	0.0	16.1
4. Lung morphometry		
a. Branching angles	Decreases with increasing depth in the lung	Increases with increasing depth in the lung
b. Symmetry	Less than humans	
c. Diameter ratio of daughter branches at bifurcation	Greater than humans	
d. Number of diversions of tracheobronchial tree	More variable than humans	
5. Mucus flow patterns	13.5 mm/min	15 mm/min
6. Bronchial glands	Absence	Numerous
7. Position of lung to ground	Horizontal	Vertical
8. Breathing	Obligate nose breathers	
C. Gut flora location	Numerous flora in stomach and proximal small intestine	Little or no flora in stomach and proximal small intestine
	Numerous flora in distal small intestine, large intestine, rectum, and feces	Similar to rat
D. Estimated β-glucuronidase activity		
Proximal small intestine	Very high (304.0 units)	0.02 units
Distal small intestine	Very high (1341.0 units)	0.09 units
E. Plasma protein binding	Generally not as extensive a binder as the human; a number of leading researchers feel that it is not possible to adequately predict the extent of human binding with the rat model.	
F. Biliary excretion	The rat is perhaps the most efficient biliary excreter, whereas limited evidence suggests that the human is not an efficient excreter of intermediately weighted compounds.	
G. Metabolism		
1. Conjugations		
Sulfate	Less active than human	
Glucuronidation	More active than human	
Acetylation	Effective	Humans display both effective and slow acetylator phenotypes
Deacetylation	Displays a relatively low ability	Human data inadequate to assess 100% conjugation with glycine
Amino acid with carboxylic acid substrates		
Benzoic acid	80%–100% conjugation with glycine	Strongly favors glutamine conjugation
Phenylacetic acid	Strongly favors glycine conjugation	

(continued)

TABLE B.14 (continued)

Comparison of Some Biochemical/Physiological/Morphological Differences of Potential Toxicological Significance between Rats and Humans

	Rat	Human
2. Rhodanese activity (liver)	Considerably more active than humans	
3. Epoxide hydrase	Less active in humans	
4. Red blood cell enzymes that prevent oxidant stress (in values relative to humans, which are given as 1)		
Glutathione peroxidase	10.2	1
Glutathione reductase	0.2	1
Catalase	0.2	1
Glucose-6-phosphate dehydrogenase	2.4	1
Superoxide dismutase	1.7	1
Methemoglobin reductase	2.4	1
5. Comparison of rat versus human for 23 substances according to qualitative and quantitative similarity of metabolic pathway	Good predictor 4 of 23; invalid predictor 8 of 23	
6. Concentration of urine	Typical laboratory rat has the ability to concentrate its urine approximately two times as much as that of humans, as indicated by urine/plasma ratios; the desert rat will concentrate its urine four to five times more than humans.	
H. Dermatotoxicity	Practical reasons preclude its widespread use as a predictive model (e.g., not sufficiently docile); the rabbit is the model of choice for historical reasons and practical considerations: docility, large skin surface, large nonpigmented eyes.	
1. Ocular		
2. Skin		
3. Allergic hypersensitivity	The rat is <i>not</i> a good model, because it does not produce anaphylactic antibodies in response to the diversity of allergens, which humans do; the guinea pig model is favored in such studies for qualitative predictions.	
I. DNA repair	In absolute terms, less efficient than humans in excision repair; when adjusted for the influence of life span, little difference between these species is found; more efficient than humans in postreplicative repair.	
J. Teratogenicity	Prolonged dependence of the rat (up to the 20–25th somite) on the inverted yolk sac placenta during organogenesis as compared with higher mammals, especially humans (5th somite), thereby making the rodent generally much more susceptible to teratogens than humans	
K. High-risk animal models		
1. Respiratory		
a. Asthma	Rats are not considered appropriate; with the exception of humans, dogs are the only animal that develop a defined hypersensitivity disease related to aeroallergens.	
b. Bronchitis	Rats are not considered an appropriate model, because of its version of chronic respiratory disease that involves bronchitis displays, excessive inflammation, and involvement of the pulmonary parenchyma.	
2. Cardiovascular		
a. Atherosclerosis	The rat is generally not considered a very effective model, because it is very resistant to developing this disease.	
b. Hypertension	Numerous predictive rat strains exist; the rat is the animal of choice.	

Source: From Calabrese, E.J., Ed., *Principles of Animal Extrapolation*, Lewis Publishers, Chelsea, MI, 1991. With permission.

REFERENCES

- Amdur, M.O. et al., Eds, *Casarett and Doull's Toxicology*, 4th edn., Pergamon Press, New York, 1991.
- Calabrese, E.J., Ed., *Principles of Animal Extrapolation*, Lewis Publishers, Chelsea, MI, 1991.
- Illing, *Xenobiotic Metabolism and Disposition: The Design of Studies on Novel Compounds*, CRC Press, Boca Raton, FL, 1989.
- Mitruka, B.M. and Rawnsley, H.M., *Clinical, Biochemical and Hematological Reference Values in Normal Experimental Animals*, Masson Publishing, New York, 1977.
- Niesink, R.J.M., de Vries, J. and Hollinger, M.A., *Toxicology: Principles and Applications*, CRC Press, Boca Raton, FL, 1996.

Appendix C: Mathematics, Symbols, Physical Constants, Conversions, and Statistics

C.1 SYMBOLS (TABLE C.1)

C.2 CONVERSIONS (TABLES C.2 THROUGH C.17)

The *Système international d'unités* (International System of Units) or SI is a modernized version of the metric system. The primary goal of the conversion to SI units is to revise the present confused measurement system and to improve test-result communications. The SI has seven basic units from which other units are derived (Table C.2):

Combinations of these base units can express any property, although for simplicity, special names are given to some of these derived units (Table C.3).

Prefixes to the base unit are used in this system to form decimal multiples and submultiples. The preferred multiples and submultiples listed in Table C.4 change the quantity by increments of 10^3 or 10^{-3} . The exceptions to these recommended factors are indicated by the asterisk.

To convert xenobiotic concentrations to or from SI units: Conversion factor (CF) = 1000/mol. wt.; conversion *to* SI units: $\mu\text{g/mL} \times \text{CF} = \mu\text{mol/L}$; conversion *from* SI units: $\mu\text{mol/L} \div \text{CF} = \mu\text{g/mL}$.

C.2.1 HOW MANY MOLECULES?

To calculate the number of molecules in any quantity of a chemical, one needs to know the weight of a mole of the chemical and the number of molecules in a mole. A mole of any chemical is its molecular weight expressed in grams. Avogadro's number, 6×10^{23} , is the number of molecules in a mole.

The number of molecules in 10 μg of benzpyrene is calculated as follows: the molecular weight of benzpyrene

is 252; therefore, a mole would weigh 252 g. 252 g is equal to $2.52 \times 10^8 \mu\text{g}$. The number of molecules in a μg is obtained by dividing the number of molecules in a mole by the number of μg in a mole. For benzpyrene, 6×10^{23} divided by 2.52×10^8 equals 2.4×10^{15} . The number of molecules in 10 μg benzpyrene would be 10 times as much, or 2.4×10^{16} .

C.3 STATISTICS (TABLES C.18 THROUGH C.25)

Statistical analysis is an integral part of a toxicology study. Advances in computer technology have allowed for more sophisticated statistical analyses to be conducted more easily and quickly on data generated in toxicology studies than would ever have been thought possible just a few decades ago. Figure C.1 presents a decision tree for choosing the proper statistical tests for the analysis of a variety of data. Although other equally valid tests may be used in place of those indicated, the scheme that is shown is a good representation of the statistical approach commonly used for assessing toxicological data. Refer to the referenced texts for a detailed discussion of the various statistical tests and their uses.

The reader should be aware of several important limitations associated with the use of statistics in toxicology: (1) statistics cannot make "poor" data "better," (2) statistical significance may not imply biological significance, (3) an effect that may have biological significance may not be statistically significant, and (4) the lack of statistical significance does not prove safety. The importance and relevance of any effect observed in a study must be assessed within the limitations imposed by the study design and the species being studied (Tables C.18 through C.24).

TABLE C.1
Greek Alphabet

Greek Letter	Greek Name	English Equivalent	Greek Letter	Greek Name	English Equivalent
A α	Alpha	a	N ν	Nu	n
B β	Beta	b	Ξ ξ	Xi	x
Γ γ	Gamma	g	Ο ο	Omicron	o
Δ δ	Delta	d	Π π	Pi	p
E ε	Epsilon	ě	Ρ ρ	Rho	r
Z ζ	Zeta	z	Σ σ ς	Sigma	s
H η	Eta	ē	Τ τ	Tau	t
Θ θ	Theta	th	Υ υ	Upsilon	u
I ι	Iota	i	Φ φ ϕ	Phi	ph
K κ	Kappa	k	Χ χ	Chi	ch
Λ λ	Lambda	l	Ψ ψ	Psi	ps
M μ	Mu	m	Ω ω	Omega	o

Note: See end of section for source.

TABLE C.2
Base Units of SI

Physical Quantity	Base Unit	SI Symbol
Length	Meter	m
Mass	Kilogram	kg
Time	Second	s
Amount of substance	Mole	mol
Thermodynamic temperature	Kelvin	K
Electric current	Ampere	A
Luminous intensity	Candela	cd

Note: See end of section for source.

TABLE C.4
Prefixes and Symbols for Decimal Multiples and Submultiples

Factor	Prefix	Symbol
10 ¹⁸	Exa	E
10 ¹⁵	Peta	P
10 ¹²	Tera	T
10 ⁹	Giga	G
10 ⁶	Mega	M
10 ³	Kilo	k
10 ^{2*}	Hecto	h
10 ^{1*}	Deka	da
10 ^{-1*}	Deci	d
10 ^{-2*}	Centi	c
10 ⁻³	Milli	m
10 ⁻⁶	Micro	μ
10 ⁻⁹	Nano	n
10 ⁻¹²	Pico	p
10 ⁻¹⁵	Femto	f
10 ⁻¹⁸	Atto	a

Note: See end of section for source.

TABLE C.3
Representative Derived Units

Derived Unit	Name and Symbol	Derivation from Base Units
Area	Square meter	m ²
Volume	Cubic meter	m ³
Force	Newton (N)	kg m/s ²
Pressure	Pascal (Pa)	kg m/s ² (N/m ²)
Work, energy	Joule (J)	kg m ² /s ² (N · m)
Mass density	Kilogram per cubic meter	kg/m ³
Frequency	Hertz (Hz)	s ⁻¹
Temperature	Degree Celsius (°C)	°C = °K – 273.15
Concentration		
Mass	Kilogram/liter	kg/L
Substance	Mole/liter	mol/L
Molality	Mole/kilogram	mol/kg
Density	Kilogram/liter	kg/L

Note: See end of section for source.

TABLE C.5**Conversion of Human Hematological Values from Traditional Units into SI Units**

Constituent	Multiplication		
	Traditional Units	Factor	SI Units
Clotting time	Minutes	0.06	ks
Prothrombin time	Seconds	1.0	arb. unit
Hematocrit (erythrocytes, volume fraction)	%	0.01	L
Hemoglobin	g/100 mL	0.6205	mmol/L
Leukocyte count (leukocytes, number concentration)	Per mm ³	10 ⁶	10 ⁹ /L
Erythrocyte count (erythrocytes, number concentration)	Million per mm ³	10 ⁶	10 ¹² /L
Mean corpuscular volume (MCV)	μ ³	1.0	fl
Mean corpuscular hemoglobin (MCH) (Erc–hemoglobin, amount of substance)	pg	0.06205	fmol
Mean corpuscular hemoglobin concentration (MCHC) (Erc–hemoglobin, substance concentration)	%	0.6205	mmol/L
Erythrocyte sedimentation rate	mm/h	1.0	arb. unit
Platelet count (blood platelets, number concentration)	mm ³	10 ⁶	10 ⁹ /L
Reticulocyte count (Erc–reticulocytes, number fraction)	% Red cells	0.01	L

Source: Young, D.S., *N. Engl. J. Med.*, 292, 795, 1975. With permission.

TABLE C.6**Conversion of Laboratory Values from Traditional Units into SI Units**

Constituent	Multiplication		
	Traditional Units	Factor	SI Units
Amylase	Units/L	1.0	arb. unit
Bilirubin (direct)	mg/100 mL	43.06	μmol/L
Conjugated	mg/100 mL	17.10	μmol/L
Total	mg/100 mL	17.10	μmol/L
Calcium	mg/100 mL	0.2495	mmol/L
Carbon dioxide	mEq/L	1.0	mmol/L
Chloride	mEq/L	1.0	mmol/L
Creatine phosphokinase (CPK)	mU/mL	0.01667	μmol/S/L
Creatinine	mg/100 mL	88.40	μmol/L
Glucose	mg/100 mL	0.05551	mmol/L
Lactic dehydrogenase	mU/mL	0.01667	μmol/S/L
Cholesterol	mg/100 mL	0.02586	mmol/L
Magnesium	mEq/L	0.50	mmol/L
P _{CO₂}	mm Hg	0.1333	kPa
pH		1.0	L
P _{O₂}	mm Hg	0.133	kPa
Phosphatase, acid	Sigma	278.4	nmol/S/L
Phosphatase, alkaline	Bodansky	0.08967	nmol/S/L
Phosphorus, inorganic	mg/100 mL	0.3229	nmol/L
Protein, total	g/100 mL	10	g/L
Protein, electrophoreses			
Albumin	% Total	0.01	L
Globulin, α ₁	% Total	0.01	L
α ₂	% Total	0.01	L
β	% Total	0.01	L
γ	% Total	0.01	L
Potassium	mEq/L	1.0	mmol/L
Sodium	mEq/L	1.0	mmol/L
Transaminase (SGOT) (aminotransferase)	Karmen	0.008051	mmol/S/L
Urea nitrogen	mg/100 mL	0.3569	mmol/L
Uric acid	mg/100 mL	0.65948	mmol/L

Source: Young, D.S., *N. Engl. J. Med.*, 292, 795, 1975. With permission.

TABLE C.7**Table for Predicting Human Half-Life of Xenobiotics from Rat Half-Life**

Rat Half-Life (H)	Lower			Human Half-Life Estimate (H)	Upper		
	95%	90%	80%		80%	90%	95%
0.01	0.019	0.025	0.034	0.106	0.327	0.451	0.598
0.02	0.034	0.045	0.062	0.189	0.574	0.790	1.045
0.03	0.048	0.064	0.087	0.264	0.799	1.098	1.450
0.04	0.062	0.081	0.111	0.335	1.011	1.387	1.830
0.05	0.074	0.098	0.134	0.404	1.213	1.664	2.193
0.06	0.087	0.114	0.156	0.469	1.408	1.930	2.543
0.07	0.099	0.130	0.178	0.533	1.598	2.189	2.882
0.08	0.110	0.146	0.199	0.596	1.782	2.441	3.213
0.09	0.122	0.161	0.220	0.657	1.963	2.687	3.536
0.1	0.13	0.18	0.24	0.72	2.14	2.93	3.85
0.2	0.24	0.31	0.43	1.27	3.78	5.17	6.79
0.3	0.34	0.44	0.60	1.78	5.28	7.21	9.47
0.4	0.43	0.56	0.77	2.26	6.70	9.14	12.00
0.5	0.51	0.68	0.92	2.72	8.05	10.99	14.42
0.6	0.60	0.79	1.07	3.17	9.36	12.77	16.76
0.7	0.68	0.89	1.22	3.60	10.63	14.51	19.04
0.8	0.76	1.00	1.36	4.02	11.88	16.20	21.26
0.9	0.84	1.10	1.50	4.44	13.09	17.86	23.44
1	0.92	1.20	1.64	4.84	14.29	19.49	25.57
2	1.63	2.13	2.91	8.60	25.40	34.66	45.47
3	2.27	2.98	4.07	12.04	35.59	48.58	63.76
4	2.88	3.78	5.16	15.28	45.24	61.76	81.09
5	3.46	4.54	6.20	18.39	54.49	74.42	97.74
6	4.02	5.28	7.21	21.39	63.46	86.69	113.88
7	4.56	5.99	8.19	24.31	72.18	98.64	129.61
8	5.08	6.68	9.14	27.15	80.71	110.32	144.99
9	5.60	7.36	10.06	29.94	89.07	121.77	160.08
10	6.1	8.0	11.0	32.7	97.3	133.0	174.9
20	10.7	14.1	19.4	58.1	174.0	238.3	313.9
30	14.9	19.7	27.0	81.3	244.6	335.6	442.4
40	18.8	24.9	34.1	103.2	311.6	427.9	564.7
50	22.6	29.8	41.0	124.1	376.1	516.9	682.7
60	26.2	34.6	47.5	144.4	438.6	603.3	797.2
70	29.6	39.2	53.9	164.1	499.5	687.5	909.1
80	33.0	43.6	60.1	183.3	559.2	770.0	1018.7
90	36.3	48.0	66.1	202.1	617.7	851.1	1126.4
100	39.5	52.3	72.0	220.6	675.2	930.8	1232.4
200	68.9	91.4	126.5	391.9	1214.2	1679.5	2230.5
300	95.2	126.7	175.7	548.6	1712.7	2374.3	3159.3
400	119.8	159.7	221.7	696.4	2186.8	3036.7	4046.6
500	143.2	191.0	265.6	837.9	2643.8	3676.1	4904.4
600	165.5	221.1	307.7	974.7	3087.5	4297.9	5739.6
700	187.1	250.1	348.5	1107.6	3520.5	4905.5	6556.7
800	208.1	278.3	388.1	1237.3	3944.6	5501.3	7358.6
900	228.5	305.8	426.8	1364.3	4361.2	6086.9	8147.4
1000	248.4	332.7	464.6	1488.9	4771.1	6663.7	8925.0
1100	267.9	359.0	501.7	1611.3	5175.1	7232.7	9692.5

Source: Bachmann, K.M. et al., *Environ. Health Perspect.*, 104, 400, 1996. With permission.

Note: The following examples indicate how this table is used. For a xenobiotic with a rat half-life of 0.8 h, the prediction or best guess of the human half-life is 4.02 h. The table indicates that the actual half-life would fall between 1.0 and 16.2 h with a confidence of 90%. Values falling between those indicated in the table can be linearly interpolated, for example, a rat half-life of 2.7 h gives a human half-life of 11.01 h.

TABLE C.8**Table for Predicting Human Volume of Distribution from Rat Volume of Distribution**

Rat Volume (L/kg)	Lower			Human Volume Estimate (L/kg)	Upper		
	95%	90%	80%		80%	90%	95%
0.01	0.002	0.003	0.004	0.011	0.031	0.041	0.054
0.02	0.004	0.005	0.007	0.020	0.057	0.076	0.099
0.03	0.006	0.008	0.011	0.029	0.082	0.109	0.141
0.04	0.008	0.010	0.014	0.038	0.105	0.141	0.182
0.05	0.010	0.013	0.017	0.046	0.128	0.172	0.222
0.06	0.012	0.015	0.020	0.055	0.151	0.202	0.261
0.07	0.013	0.017	0.023	0.063	0.174	0.232	0.299
0.08	0.015	0.019	0.026	0.071	0.196	0.261	0.337
0.09	0.017	0.022	0.029	0.079	0.217	0.290	0.374
0.1	0.019	0.024	0.032	0.087	0.239	0.319	0.411
0.2	0.035	0.045	0.060	0.164	0.445	0.593	0.762
0.3	0.051	0.065	0.087	0.236	0.641	0.853	1.096
0.4	0.066	0.085	0.113	0.307	0.831	1.105	1.419
0.5	0.081	0.104	0.139	0.376	1.016	1.352	1.735
0.6	0.096	0.123	0.164	0.443	1.198	1.593	2.045
0.7	0.111	0.142	0.189	0.510	1.377	1.832	2.350
0.8	0.125	0.160	0.213	0.575	1.554	2.067	2.652
0.9	0.139	0.178	0.237	0.640	1.729	2.299	2.950
1	0.15	0.20	0.26	0.70	1.90	2.53	3.25
2	0.29	0.37	0.49	1.32	3.57	4.75	6.09
3	0.41	0.53	0.70	1.91	5.16	6.87	8.82
4	0.53	0.69	0.91	2.48	6.71	8.93	11.47
5	0.65	0.84	1.18	3.03	8.23	10.96	14.08
6	0.77	0.99	1.32	3.58	9.72	12.95	16.64
7	0.88	1.14	1.51	4.11	11.19	14.92	19.17
8	0.99	1.28	1.71	4.64	12.65	16.86	21.68
9	1.11	1.42	1.90	5.17	14.09	18.79	24.17
10	1.21	1.56	2.08	5.69	15.52	20.70	26.63
20	2.25	2.90	3.88	10.66	29.32	39.20	50.54
30	3.22	4.16	5.57	15.40	42.60	57.04	73.63
40	4.15	5.37	7.20	19.99	55.54	74.46	96.23
50	5.06	6.54	8.78	24.48	68.24	91.59	118.48
60	5.94	7.69	10.33	28.88	80.77	108.50	140.45
70	6.80	8.81	11.84	33.22	93.14	125.22	162.21
80	7.65	9.91	13.34	37.49	105.39	141.79	183.79
90	8.48	11.00	14.81	41.72	117.54	158.23	205.22

Source: Bachmann, K.M. et al., *Environ. Health Perspect.*, 104, 400, 1996. With permission.

Note: See note of Table C.7 for examples of how this table is used.

TABLE C.9
Approximate Metric and Apothecary Weight Equivalents

Metric	Apothecary	Metric	Apothecary
1 gram (g)	= 15 grains	0.05 g (50 mg)	= 3/4 grain
0.6 g (600 mg)	= 10 grains	0.03 g (30 mg)	= 1/2 grain
0.5 g (500 mg)	= 7½ grains	0.015 g (15 mg)	= 1/4 grain
0.3 g (300 mg)	= 5 grains	0.001 g (1 mg)	= 1/80 grain
0.2 g (200 mg)	= 3 grains	0.6 mg	= 1/100 grain
0.1 g (100 mg)	= 1½ grains	0.5 mg	= 1/120 grain
0.06 g (60 mg)	= 1 grain	0.4 mg	= 1/150 grain

Approximate Household, Apothecary, and Metric Volume Equivalents

Household	Apothecary	Metric
1 teaspoon (t or tsp)	= 1 fluidram (f ₃)	= 5 mL ^{a,b}
1 tablespoon (T or tbs)	= ½ fluidounce (f ₃)	= 15 mL
2 tablespoons	= 1 fluidounce	= 30 mL
1 measuring cupful	= 8 fluidounces	= 240 mL
1 pint (pt)	= 16 fluidounces	= 473 mL
1 quart (qt)	= 32 fluidounces	= 946 mL
1 gallon (gal)	= 128 fluidounces	= 3785 mL

Note: See end of section for source.

^a 1 mL = 1 cubic centimeter (cc); however, mL is the preferred measurement term today.

^b 1 teaspoon (metric) = 5 mL, 1 teaspoon (United States) = 4.9 mL, 1 teaspoon (United Kingdom) = 3.6 mL.

TABLE C.10
Conversion Factors: Metric to English

To Obtain	Multiply	By
Inches	Centimeters	0.3937007874
Feet	Meters	3.280839895
Yards	Meters	1.093613298
Miles	Kilometers	0.6213711922
Ounces	Grams	$3.527396195 \times 10^{-2}$
Pounds	Kilograms	2.204622622
Gallons (US liquid)	Liters	0.2641720524
Fluid ounces	Milliliters (cc)	$3.381402270 \times 10^{-2}$
Square inches	Square centimeters	0.1550003100
Square feet	Square meters	10.76391042
Square yards	Square meters	1.195990046
Cubic inches	Milliliters (cc)	$6.102374409 \times 10^{-2}$
Cubic feet	Cubic meters	35.31466672
Cubic yards	Cubic meters	1.307950619

Note: See end of section for source.

TABLE C.11
Conversion Factors: English to Metric^a

To Obtain	Multiply	By
Centimeters	Inches	2.54
Meters	Feet	0.3048
Meters	Yards	0.9144
Kilometers	Miles	1.609344
Grains	Ounces	28.34952313
Kilograms	Pounds	0.45359237
Liters	Gallons (US liquid)	3.785411784
Milliliters (cc)	Fluid ounces	29.57352956
Square centimeters	Square inches	6.4516
Square meters	Square feet	0.09290304
Square meters	Square yards	0.83612736
Milliliters (cc)	Cubic inches	16.387064
Cubic meters	Cubic feet	$2.831684659 \times 10^{-2}$
Cubic meters	Cubic yards	0.764554858

^a Boldface numbers are exact; others are given to 10 significant figures where so indicated by the multiplier factor. See end of section for source.

TABLE C.12
Conversion Factors: General^a

To Obtain	Multiply	By
Atmospheres	Feet of water at 4°C	2.950×10^{-2}
Atmospheres	Inches of mercury at 0°C	3.342×10^{-2}
Atmospheres	Pounds per square inch	6.804×10^{-2}
Pascals	Millibars	1×10^2
Pascals	Millimeters of mercury at 0°C	1.33×10^2
Pascals	Pounds per square inch	6.894×10^3
BTU	Foot-pounds	1.285×10^{-3}
BTU	Joules	9.480×10^{-4}
Cubic feet	Cords	128
Degrees (angle)	Radians	57.2958
Ergs	Foot-pounds	1.356×10^7
Feet	Miles	5280
Feet of water at 40°C	Atmospheres	33.90
Foot-pounds	Horsepower-hours	1.98×10^6
Foot-pounds	Kilowatt-hours	2.655×10^6
Foot-pounds per minute	Horsepower	3.3×10^4
Horsepower	Foot-pounds per second	1.818×10^{-3}
Inches of mercury at 0°C	Pounds per square inch	2.036
Joules	BTU	1.054.8
Joules	Foot-pounds	1.35582
Kilowatts	BTU per minute	1.758×10^{-2}
Kilowatts	Foot-pounds per minute	2.26×10^{-5}
Kilowatts	Horsepower	0.745712
Knots	Miles per hour	0.86897624
Miles	Feet	1.894×10^{-4}
Nautical miles	Miles	0.86897624
Radians	Degrees	1.745×10^{-2}
Square feet	Acres	43,560
Watts	BTU per minute	17.5796

Note: See www.onlineconversion.com for other conversions.

^a Boldface numbers are exact; others are given to 10 significant figures where so indicated by the multiplier factor. See end of section for source.

TABLE C.13
Temperature Factors^a

$$F = 9/5 (^{\circ}\text{C}) + 32$$

$$\text{Fahrenheit temperature} = 1.8 (\text{temperature in kelvins}) - 459.67$$

$$^{\circ}\text{C} = 5/9 [(^{\circ}\text{F}) - 32]$$

$$\text{Celsius temperature} = \text{temperature in kelvins} - 273.15$$

$$\text{Fahrenheit temperature} = 1.8 (\text{Celsius temperature}) + 32$$

Conversion of Temperatures To	
From	
$^{\circ}\text{Celsius}$	$^{\circ}\text{Fahrenheit}$ $t_F = (t_c \times 1.8) + 32$
	Kelvin $T_K = t_c + 273.15$
	$^{\circ}\text{Rankine}$ $T_R = (t_c + 273.15) \times 1.8$
$^{\circ}\text{Fahrenheit}$	$^{\circ}\text{Celsius}$ $T_Y = \frac{t_F - 32}{1.8}$
	Kelvin $T_K = \frac{t_F - 32}{1.8} + 273.15$
	$^{\circ}\text{Rankine}$ $T_R = t_F + 459.67$
Kelvin	$^{\circ}\text{Celsius}$ $t_c = T_K - 273.15$
	$^{\circ}\text{Rankine}$ $T_R = T_K \times 1.8$
$^{\circ}\text{Rankine}$	$^{\circ}\text{Fahrenheit}$ $t_F = T_R - 459.67$
	Kelvin $T_K = \frac{T_R}{1.8}$

^a Boldface numbers are exact; others are given to 10 significant figures where so indicated by the multiplier factor. See end of section for source.

TABLE C.14
Temperature Conversions

$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$
-10	-23.3	35	+1.6	85	29.4	135	57.2	185	85.0
-5	-20.5	40	4.4	90	32.2	140	60.0	190	87.8
0	-17.8	45	7.2	95	35.0	145	62.8	195	90.5
+5	-15.0	50	10.0	100	37.8	150	65.5	200	93.3
10	-12.2	55	12.8	105	40.5	155	68.3	205	96.1
15	-9.4	60	15.5	110	43.3	160	71.1	210	98.9
20	-6.6	65	18.3	115	46.1	165	73.9	212	100
25	-3.9	70	21.1	120	48.9	170	76.6		
30	-1.1	75	23.9	125	51.6	175	79.4		
32	0	80	26.6	130	54.4	180	82.2		

TABLE C.15
Table of Equivalents

kg	= 1000 g, 1 million mg, 2.2 lbs
g	= 1000 mg, 1 million μg , approx. 0.035 oz
mg	= 1000 μg , 1 million ng
μg	= 1000 ng
L	= approx. 1 quart, approx. 33 oz
lb	= 16 oz, 454.5 g, 0.45 kg
oz	= 28.4 g
acre	= 4047 m^2
hectare	= 2.5 acres

When referring to the concentration of a chemical in food or other medium:

mg/kg	= ppm, $\mu\text{g/g}$
mg/L	= ppm = 0.0001 %
$\mu\text{g/kg}$	= ppb, ng/g
ng/kg	= ppt
ppm	= mg/kg, $\mu\text{g/g}$
ppb	= $\mu\text{g/kg}$, ng/g
ppt	= ng/kg

Note: See end of section for source.

TABLE C.16
Overview of Units Used to Express the Concentration of a Substance

Compartment	Units	Abbreviation	Conversion ^a
Air (gases)	$\mu\text{g}/\text{m}^3$		1 $\mu\text{g}/\text{m}^3$ is V/A $\mu\text{L}/\text{m}^3$
	$\mu\text{mol}/\text{m}^3$		1 $\mu\text{mol}/\text{m}^3$ is V $\mu\text{L}/\text{m}^3$
	$\mu\text{L}/\text{m}^3$	ppbv	
	μL^{-1}	ppmv	
Water	$\mu\text{g}/\text{L}$	ppb	
	mg/L	ppm	
	$\mu\text{mol}/\text{L}$	μM	1 $\mu\text{mol}/\text{L}$ is A $\mu\text{g}/\text{L}$
Soil	$\mu\text{g}/\text{kg}$	ppb	
	mg/kg	ppm	
	$\mu\text{g}/\text{g}$	ppm	
	$\mu\text{mol}/\text{g}$		1 $\mu\text{mol}/\text{g}$ is A $\mu\text{g}/\text{g}$

Note: See end of section for source.

^a A = molecular weight of the substance; V = molar volume at current pressure and temperature.

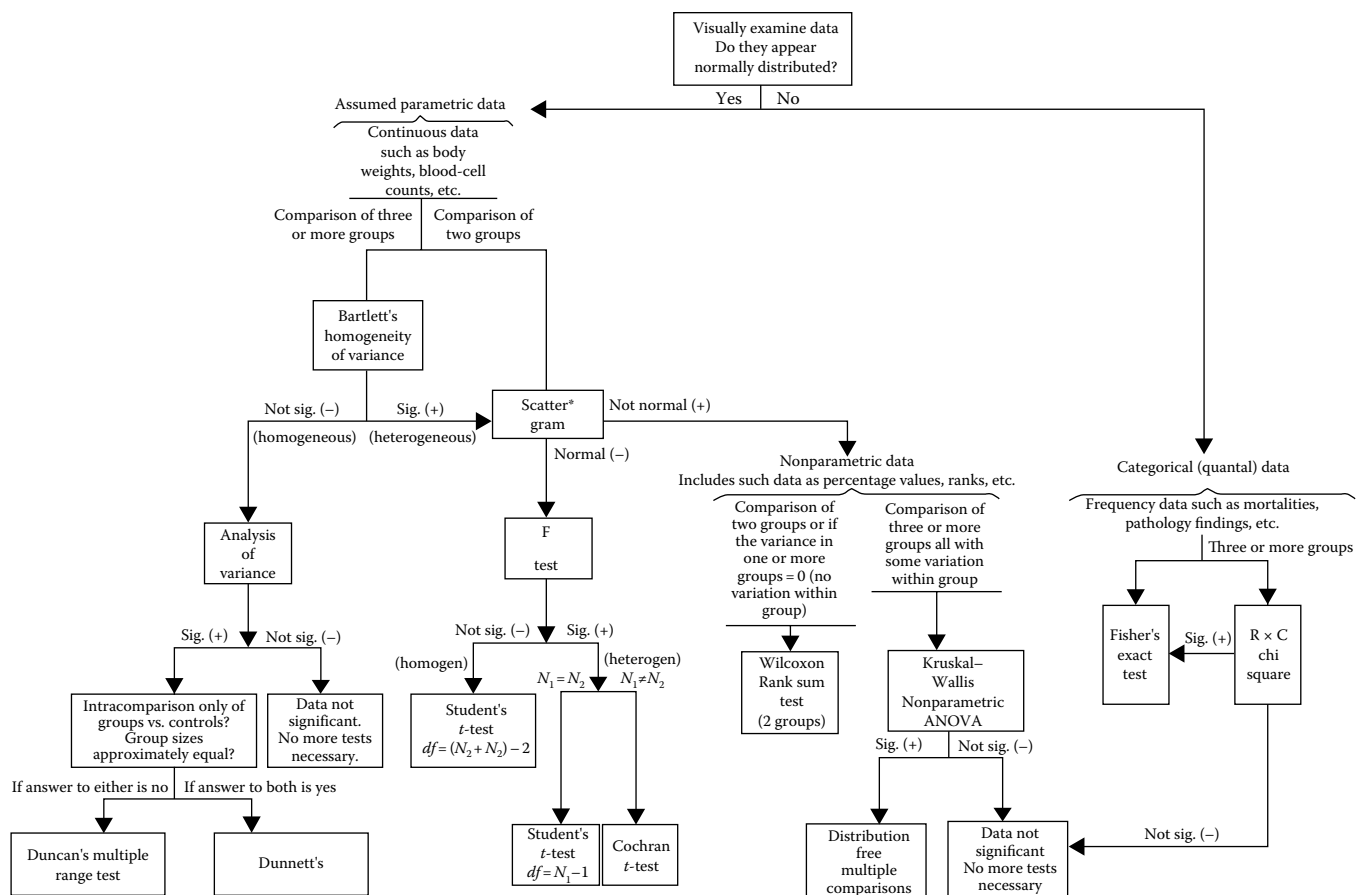
TABLE C.17**Conversion of Radioactivity Units from mCi and μ Ci to MBq**

mCi	MBq	mCi	MBq	μCi	MBq	mCi	MBq
200	7400	10	370	1000	37.0	80	2.96
150	5550	9	333	900	33.3	70	2.59
100	3700	8	296	800	29.6	60	2.22
90	3330	7	259	700	25.9	50	1.85
80	2960	6	222	600	22.2	40	1.48
70	2590	5	185	500	18.5	30	1.11
60	2220	4	148	400	14.8	20	0.74
50	1850	3	111	300	11.1	10	0.37
40	1480	2	74.0	200	7.4	5	0.185
30	1110	1	37.0	100	3.7	2	0.074
20	740			90	3.33	1	0.037

Notes: The unit of radioactivity in the International System of Units (SI) is the becquerel, which is equal to one nuclear transformation per second. See end of section for source.

Conversion factors are

1 becquerel (Bq)	= 1 nuclear transformation per second
1 curie (Ci)	= 3.7×10^{10} becquerels (exactly)
	= 37 gigabecquerels (GBq)
1 millicurie (mCi)	= 3.7×10^7 becquerels
	= 37 megabecquerels (MBq)
1 microcurie (μ Ci)	= 3.7×10^4 becquerels
	= 37 kilobecquerels (kBq)
1 gigabecquerel (GBq)	= 27.027 millicuries (mCi)
1 megabecquerel (MBq)	= 27.027 microcuries (μ Ci)
1 kilobecquerel (kBq)	= 27.027 nanocuries (nCi)



* If plot does not clearly demonstrate lack of normality exact tests may be employed.

- If continuous data, Kolmogorov-Smirnov test.

- If discontinuous data, chi-square goodness-of-fit test may be used.

FIGURE C.1 Decision tree for selecting hypothesis-testing procedures. Dunn's summed rank test is generally performed after the Kruskal-Wallis ANOVA for multiple comparisons of nonparametric data. For trend analysis of parametric data, Jonckheere test for monotonic trend can be used. (From Gad, S. and Weil, C.S., in *Statistics and Experimental Design for Toxicologists*, Telford Press, Caldwell, NJ, 1986. With permission.)

TABLE C.18
Transformation of Percentages into Logits

Percentage	0	1	2	3	4	5	6	7	8	9
50	0	0.04	0.08	0.12	0.16	0.20	0.24	0.28	0.32	0.36
60	0.41	0.45	0.49	0.53	0.58	0.62	0.66	0.71	0.75	0.80
70	0.85	0.90	0.94	0.99	1.05	1.10	1.15	1.21	1.27	1.32
80	1.38	1.45	1.52	1.59	1.66	1.73	1.82	1.90	1.99	2.09
90	2.20	2.31	2.44	2.59	2.75	2.94	3.18	3.48	3.89	4.60
99	4.60	4.70	4.82	4.95	5.11	5.29	5.52	5.81	6.21	6.91

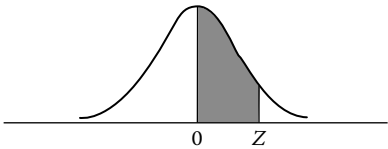
Note: See end of section for source.

TABLE C.19
Transformation of Percentages into Probits

Percentage	0	1	2	3	4	5	6	7	8	9
0	[−]	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.07

Note: See end of section for source.

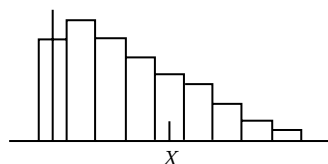
TABLE C.20
Areas under the Standard Normal Curve



Z	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.0	0.0000	0.0040	0.0080	0.0120	0.0160	0.0199	0.0239	0.0279	0.0319	0.0359
0.1	0.0398	0.0438	0.0478	0.0517	0.0557	0.0596	0.0636	0.0675	0.0714	0.0753
0.2	0.0793	0.0832	0.0871	0.0910	0.0948	0.0987	0.1026	0.1064	0.1103	0.1141
0.3	0.1179	0.1217	0.1255	0.1293	0.1331	0.1368	0.1406	0.1443	0.1480	0.1517
0.4	0.1554	0.1591	0.1628	0.1664	0.1700	0.1736	0.1772	0.1808	0.1844	0.1879
0.5	0.1915	0.1950	0.1985	0.2019	0.2054	0.2088	0.2123	0.2157	0.2190	0.2224
0.6	0.2257	0.2291	0.2324	0.2357	0.2389	0.2422	0.2454	0.2486	0.2517	0.2549
0.7	0.2580	0.2611	0.2642	0.2673	0.2704	0.2734	0.2764	0.2794	0.2823	0.2852
0.8	0.2881	0.2910	0.2939	0.2967	0.2995	0.3023	0.3051	0.3078	0.3106	0.3133
0.9	0.3159	0.3186	0.3212	0.3238	0.3264	0.3289	0.3315	0.3340	0.3365	0.3389
1.0	0.3413	0.3438	0.3461	0.3485	0.3508	0.3531	0.3554	0.3577	0.3599	0.3621
1.1	0.3643	0.3665	0.3686	0.3708	0.3729	0.3749	0.3770	0.3790	0.3810	0.3830
1.2	0.3849	0.3869	0.3888	0.3907	0.3925	0.3944	0.3962	0.3980	0.3997	0.4015
1.3	0.4032	0.4049	0.4066	0.4082	0.4099	0.4115	0.4131	0.4147	0.4162	0.4177
1.4	0.4192	0.4207	0.4222	0.4236	0.4251	0.4265	0.4279	0.4292	0.4306	0.4319
1.5	0.4332	0.4345	0.4357	0.4370	0.4382	0.4394	0.4406	0.4418	0.4429	0.4441
1.6	0.4452	0.4463	0.4474	0.4484	0.4495	0.4505	0.4515	0.4525	0.4535	0.4545
1.7	0.4554	0.4564	0.4573	0.4582	0.4591	0.4599	0.4608	0.4616	0.4625	0.4633
1.8	0.4641	0.4649	0.4656	0.4664	0.4671	0.4678	0.4686	0.4693	0.4699	0.4706
1.9	0.4713	0.4719	0.4726	0.4732	0.4738	0.4744	0.4750	0.4756	0.4761	0.4767
2.0	0.4772	0.4778	0.4783	0.4788	0.4793	0.4798	0.4803	0.4808	0.4812	0.4817
2.1	0.4821	0.4826	0.4830	0.4834	0.4838	0.4842	0.4846	0.4850	0.4854	0.4857
2.2	0.4861	0.4864	0.4868	0.4871	0.4875	0.4878	0.4881	0.4884	0.4887	0.4890
2.3	0.4893	0.4896	0.4898	0.4901	0.4904	0.4906	0.4909	0.4911	0.4913	0.4916
2.4	0.4918	0.4920	0.4922	0.4925	0.4927	0.4929	0.4931	0.4932	0.4934	0.4936
2.5	0.4938	0.4940	0.4941	0.4943	0.4945	0.4946	0.4948	0.4949	0.4951	0.4952
2.6	0.4953	0.4955	0.4956	0.4957	0.4959	0.4960	0.4961	0.4962	0.4963	0.4964
2.7	0.4965	0.4966	0.4967	0.4968	0.4969	0.4970	0.4971	0.4972	0.4973	0.4974
2.8	0.4974	0.4975	0.4976	0.4977	0.4977	0.4978	0.4979	0.4979	0.4980	0.4981
2.9	0.4981	0.4982	0.4982	0.4983	0.4984	0.4984	0.4985	0.4985	0.4986	0.4986
3.0	0.4987	0.4987	0.4987	0.4988	0.4988	0.4989	0.4989	0.4989	0.4990	0.4990

Note: See end of section for source.

TABLE C.21
Poisson Distribution

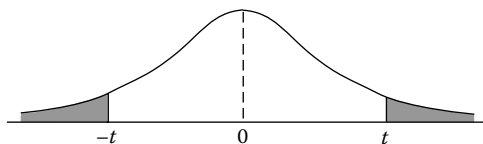


Each number in this table represents the probability of obtaining at least X successes, or the area under the histogram to the right of and including the rectangle whose center is at X .

m	$X = 0$	$X = 1$	$X = 2$	$X = 3$	$X = 4$	$X = 5$	$X = 6$	$X = 7$	$X = 8$	$X = 9$	$X = 10$	$X = 11$	$X = 12$	$X = 13$	$X = 14$
0.10	1.000	0.095	0.005												
0.20	1.000	0.181	0.018	0.001											
0.30	1.000	0.259	0.037	0.004											
0.40	1.000	0.330	0.062	0.008	0.001										
0.50	1.000	0.393	0.090	0.014	0.002										
0.60	1.000	0.451	0.122	0.023	0.003										
0.70	1.000	0.503	0.156	0.034	0.006	0.001									
0.80	1.000	0.551	0.191	0.047	0.009	0.001									
0.90	1.000	0.593	0.228	0.063	0.013	0.002									
1.00	1.000	0.632	0.264	0.080	0.019	0.004	0.001								
1.1	1.000	0.667	0.301	0.100	0.026	0.005	0.001								
1.2	1.000	0.699	0.337	0.120	0.034	0.008	0.002								
1.3	1.000	0.727	0.373	0.143	0.043	0.011	0.002								
1.4	1.000	0.753	0.408	0.167	0.054	0.014	0.003	0.001							
1.5	1.000	0.777	0.442	0.191	0.066	0.019	0.004	0.001							
1.6	1.000	0.798	0.475	0.217	0.079	0.024	0.006	0.001							
1.7	1.000	0.817	0.507	0.243	0.093	0.030	0.008	0.002							
1.8	1.000	0.835	0.537	0.269	0.109	0.036	0.010	0.003	0.001						
1.9	1.000	0.850	0.566	0.296	0.125	0.044	0.013	0.003	0.001						
2.0	1.000	0.865	0.594	0.323	0.143	0.053	0.017	0.005	0.001						
2.2	1.000	0.889	0.645	0.377	0.181	0.072	0.025	0.007	0.002						
2.4	1.000	0.909	0.692	0.430	0.221	0.096	0.036	0.012	0.003	0.001					
2.6	1.000	0.926	0.733	0.482	0.264	0.123	0.049	0.017	0.005	0.001					
2.8	1.000	0.939	0.769	0.531	0.308	0.152	0.065	0.024	0.008	0.002	0.001				
3.0	1.000	0.950	0.801	0.577	0.353	0.185	0.084	0.034	0.012	0.004	0.001				
3.2	1.000	0.959	0.829	0.620	0.397	0.219	0.105	0.045	0.017	0.006	0.002				
3.4	1.000	0.967	0.853	0.660	0.442	0.256	0.129	0.058	0.023	0.008	0.003	0.001			
3.6	1.000	0.973	0.874	0.697	0.485	0.294	0.156	0.073	0.031	0.012	0.004	0.001			
3.8	1.000	0.978	0.893	0.731	0.527	0.332	0.184	0.091	0.040	0.016	0.006	0.002			
4.0	1.000	0.982	0.908	0.762	0.567	0.371	0.215	0.111	0.051	0.021	0.008	0.003	0.001		
4.2	1.000	0.985	0.922	0.790	0.605	0.410	0.247	0.133	0.064	0.028	0.011	0.004	0.001		
4.4	1.000	0.988	0.934	0.815	0.641	0.449	0.280	0.156	0.079	0.036	0.015	0.006	0.002	0.001	
4.6	1.000	0.990	0.944	0.837	0.674	0.487	0.314	0.182	0.095	0.045	0.020	0.008	0.003	0.001	
4.8	1.000	0.992	0.952	0.857	0.706	0.524	0.349	0.209	0.113	0.056	0.025	0.010	0.004	0.001	
5.0	1.000	0.993	0.960	0.875	0.735	0.560	0.384	0.238	0.133	0.068	0.032	0.014	0.005	0.002	0.001

Note: See end of section for source.

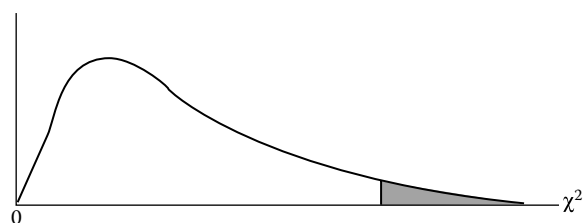
TABLE C.22
***t* Distribution**



Degrees of Freedom, <i>f</i>	90% (<i>P</i> = 0.1)	95% (<i>P</i> = 0.05)	99% (<i>P</i> = 0.01)
1	6.314	12.706	63.657
2	2.920	4.303	9.925
3	2.353	3.182	5.841
4	2.132	2.776	4.604
5	2.015	2.571	4.032
6	1.943	2.447	3.707
7	1.895	2.365	3.499
8	1.860	2.306	3.355
9	1.833	2.262	3.250
10	1.812	2.228	3.169
11	1.796	2.201	3.106
12	1.782	2.179	3.055
13	1.771	2.160	3.012
14	1.761	2.145	2.977
15	1.753	2.131	2.947
16	1.746	2.120	2.921
17	1.740	2.110	2.898
18	1.734	2.101	2.878
19	1.729	2.093	2.861
20	1.725	2.086	2.845
21	1.721	2.080	2.831
22	1.717	2.074	2.819
23	1.714	2.069	2.807
24	1.711	2.064	2.797
25	1.708	2.060	2.787
26	1.706	2.056	2.779
27	1.703	2.052	2.771
28	1.701	2.048	2.763
29	1.699	2.045	2.756
inf.	1.645	1.960	2.576

Note: See end of section for source.

TABLE C.23
 χ^2 Distribution



ν	0.05	0.025	0.01	0.005
1	3.841	5.024	6.635	7.879
2	5.991	7.378	9.210	10.597
3	7.815	9.348	11.345	12.838
4	9.488	11.143	13.277	14.860
5	11.070	12.832	15.086	16.750
6	12.592	14.449	16.812	18.548
7	14.067	16.013	18.475	20.278
8	15.507	17.535	20.090	21.955
9	16.919	19.023	21.666	23.589
10	18.307	20.483	23.209	25.188
11	19.675	21.920	24.725	26.757
12	21.026	23.337	26.217	28.300
13	22.362	24.736	27.688	29.819
14	23.685	26.119	29.141	31.319
15	24.996	27.488	30.578	32.801
16	26.296	28.845	32.000	34.267
17	27.587	30.191	33.409	35.718
18	28.869	31.526	34.805	37.156
19	30.144	32.852	36.191	38.582
20	31.410	34.170	37.566	39.997
21	32.671	35.479	38.932	41.401
22	33.924	36.781	40.289	42.796
23	35.172	38.076	41.638	44.181
24	36.415	39.364	42.980	45.558
25	37.652	40.646	44.314	46.928
26	38.885	41.923	45.642	48.290
27	40.113	43.194	46.963	49.645
28	41.337	44.461	48.278	50.993
29	42.557	45.722	49.588	52.336
30	43.773	46.979	50.892	53.672

Note: See end of section for source.

TABLE C.24
Variance Ratio

F (95%)

n_2	n_1									
	1	2	3	4	5	6	8	12	24	∞
1	161.4	199.5	215.7	224.6	230.2	234.0	238.9	243.9	249.0	254.3
2	18.51	19.00	19.16	19.25	19.30	19.33	19.37	19.41	19.45	19.50
3	10.13	9.55	9.28	9.12	9.01	8.94	8.84	8.74	8.64	8.53
4	7.71	6.94	6.59	6.39	6.26	6.16	6.04	5.91	5.77	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.82	4.68	4.53	4.36
6	5.99	5.14	4.76	4.53	4.39	4.28	4.15	4.00	3.84	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.73	3.57	3.41	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.44	3.28	3.12	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.23	3.07	2.90	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.07	2.91	2.74	2.54
11	4.84	3.98	3.59	3.36	3.20	3.09	2.95	2.79	2.61	2.40
12	4.75	3.88	3.49	3.26	3.11	3.00	2.85	2.69	2.50	2.30
13	4.67	3.80	3.41	3.18	3.02	2.92	2.77	2.60	2.42	2.21
14	4.60	3.74	3.34	3.11	2.96	2.85	2.70	2.53	2.35	2.13
15	4.54	3.68	3.29	3.06	2.90	2.79	2.64	2.48	2.29	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.59	2.42	2.24	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.55	2.38	2.19	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.51	2.34	2.15	1.92
19	4.38	3.52	3.13	2.90	2.74	2.63	2.48	2.31	2.11	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.45	2.28	2.08	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.42	2.25	2.05	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.40	2.23	2.03	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.38	2.20	2.00	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.36	2.18	1.98	1.73
25	4.24	3.38	2.99	2.76	2.60	2.49	2.34	2.16	1.96	1.71
26	4.22	3.37	2.98	2.74	2.59	2.47	2.32	2.15	1.95	1.69
27	4.21	3.35	2.96	2.73	2.57	2.46	2.30	2.13	1.93	1.67
28	4.20	3.34	2.95	2.71	2.56	2.44	2.29	2.12	1.91	1.65
29	4.18	3.33	2.93	2.70	2.54	2.43	2.28	2.10	1.90	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.27	2.09	1.89	1.62
40	4.08	3.23	2.84	2.61	2.45	2.34	2.18	2.00	1.79	1.51
60	4.00	3.15	2.76	2.52	2.37	2.25	2.10	1.92	1.70	1.39
120	3.92	3.07	2.68	2.45	2.29	2.17	2.02	1.83	1.61	1.25
∞	3.84	2.99	2.60	2.37	2.21	2.10	1.94	1.75	1.52	1.00

F (99%)

1	4052	4999	5403	5625	5764	5859	5982	6106	6234	6366
2	98.50	99.00	99.17	99.25	99.30	99.33	99.37	99.42	99.46	99.50
3	34.12	30.82	29.46	28.71	28.24	27.91	27.49	27.05	26.60	26.12
4	21.20	18.00	16.69	15.98	15.52	15.21	14.80	14.37	13.93	13.46
5	16.26	13.27	12.06	11.39	10.97	10.67	10.29	9.89	9.47	9.02
6	13.74	10.92	9.78	9.15	8.75	8.47	8.10	7.72	7.31	6.88
7	12.25	9.55	8.45	7.85	7.46	7.19	6.84	6.47	6.07	5.65
8	11.26	8.65	7.59	7.01	6.63	6.37	6.03	5.67	5.28	4.86
9	10.56	8.02	6.99	6.42	6.06	5.80	5.47	5.11	4.73	4.31
10	10.04	7.56	6.55	5.99	5.64	5.39	5.06	4.71	4.33	3.91
11	9.65	7.20	6.22	5.67	5.32	5.07	4.74	4.40	4.02	3.60
12	9.33	6.93	5.95	5.41	5.06	4.82	4.50	4.16	3.78	3.36
13	9.07	6.70	5.74	5.20	4.86	4.62	4.30	3.96	3.59	3.16
14	8.86	6.51	5.56	5.03	4.69	4.46	4.14	3.80	3.43	3.00

TABLE C.24 (continued)
Variance Ratio

F (99%)

n_2	n_1									
	1	2	3	4	5	6	8	12	24	∞
15	8.68	6.36	5.42	4.89	4.56	4.32	4.00	3.67	3.29	2.87
16	8.53	6.23	5.29	4.77	4.44	4.20	3.89	3.55	3.18	2.75
17	8.40	6.11	5.18	4.67	4.34	4.10	3.79	3.45	3.08	2.65
18	8.28	6.01	5.09	4.58	4.25	4.01	3.71	3.37	3.00	2.57
19	8.18	5.93	5.01	4.50	4.17	3.94	3.63	3.30	2.92	2.49
20	8.10	5.85	4.94	4.43	4.10	3.87	3.56	3.23	2.86	2.42
21	8.02	5.78	4.87	4.37	4.04	3.81	3.51	3.17	2.80	2.36
22	7.94	5.72	4.82	4.31	3.99	3.76	3.45	3.12	2.75	2.31
23	7.88	5.66	4.76	4.26	3.94	3.71	3.41	3.07	2.70	2.26
24	7.82	5.61	4.72	4.22	3.90	3.67	3.36	3.03	2.66	2.21
25	7.77	5.57	4.68	4.18	3.86	3.63	3.32	2.99	2.62	2.17
26	7.72	5.53	4.64	4.14	3.82	3.59	3.29	2.96	2.58	2.13
27	7.68	5.49	4.60	4.11	3.78	3.56	3.26	2.93	2.55	2.10
28	7.64	5.45	4.57	4.07	3.75	3.53	3.23	2.90	2.52	2.06
29	7.60	5.42	4.54	4.04	3.73	3.50	3.20	2.87	2.49	2.03
30	7.56	5.39	4.51	4.02	3.70	3.47	3.17	2.84	2.47	2.01
40	7.31	5.18	4.31	3.83	3.51	3.29	2.99	2.66	2.29	1.80
60	7.08	4.98	4.13	3.65	3.34	3.12	2.82	2.50	2.12	1.60
120	6.85	4.79	3.95	3.48	3.17	2.96	2.66	2.34	1.95	1.38
∞	6.64	4.60	3.78	3.32	3.02	2.80	2.51	2.18	1.79	1.00

Note: See end of section for source.

GENERAL STATISTICAL REFERENCES

- Adler, H. L. and Roessler, E. B., *Introduction to Probability and Statistics*, 6th edn., H. Freeman, New York, 1977.
- Gad, S. and Weil, C. S., *Statistics and Experimental Design for Toxicologists*, Telford Press, Caldwell, NJ, 1986.
- Hollander, M. and Wolfe, D. A., *Nonparametric Statistical Methods*, John Wiley & Sons, New York, 1973.
- Snedecor, G. W. and Cochran, W. G., *Statistical Methods*, 6th edn., Iowa State University Press, Ames, IA, 1967.
- Tallarida, R. J. and Murray, R. B., *Manual of Pharmacologic Calculations with Computer Programs*, 2nd edn., Springer-Verlag, New York, 1987.

MATHEMATICS, SYMBOLS, PHYSICAL CONSTANTS, CONVERSIONS, AND STATISTICS

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- Beyer, W. H., Ed., *CRC Standard Mathematical Tables and Formulae*, 29th edn., CRC Press, Boca Raton, FL, 1991: Greek alphabet,

conversion constants and multipliers (recommended decimal multiples and submultiples, metric to English, English to metric, general, temperature factors), physical constants, series expansion, integrals, the Fourier transforms, numerical methods, probability, positional notation.

- Lide, D. R., Ed., *CRC Handbook of Chemistry and Physics*, 73rd edn., CRC Press, Boca Raton, FL, 1992: International System of Units (SI), conversion constants and multipliers (conversion of temperatures), symbols and terminology for physical and chemical quantities, fundamental physical constants.
- Pankow, J. F., *Aquatic Chemistry Concepts*, Lewis Publishers, Chelsea, MI, 1991.
- Shackelford, J. and Alexander, W. Eds., *CRC Materials Science and Engineering Handbook*, CRC Press, Boca Raton, FL, 1992.
- Tallarida, R. J., *Pocket Book of Integrals and Mathematical Formulas*, 2nd edn., CRC Press, Boca Raton, FL, 1992: Statistics; tables of probability and statistics; table of derivatives.

Appendix D: Calculations, Preparation, and Properties of Substances Used in Toxicology

D.1 MOLARITY, MOLALITY, NORMALITY, OSMOLARITY CALCULATIONS

$$\text{Molarity (M)} = \frac{\text{Number of moles of solute}}{\text{Liter of solution}}$$

$$\text{where Number of moles} = \frac{\text{Grams of chemical}}{\text{Molecular weight}}$$

$$\text{Molality (m)} = \frac{\text{Number of moles of solute}}{\text{Kilogram of solution}}$$

$$\text{Normality (N)} = \frac{\text{Number of equivalents of solute}}{\text{Liter of solution}}$$

$$\text{where Number of equivalents} = \frac{\text{Grams of chemical}}{\text{Equivalent weight}}$$

$$\text{Equivalent weight} = \frac{\text{Molecular weight}}{n}$$

For acids and bases, n is the number of replaceable H^+ or OH^- ions per molecule.

$$\text{Normality} = n \text{ Molarity}$$

where n is the number of replaceable H^+ or OH^- ions per molecule.

$$\text{Osmolarity} = n \text{ Molarity}$$

where n is the number of dissociable ions per molecule.

D.2 SOLUTION CALCULATIONS

$$\text{Volume percent (\% v/v)} = \frac{\text{Volume of solute}}{\text{Volume of solution}} \times 100$$

$$\text{Weight percent (\% w/w)} = \frac{\text{Weight of solute}}{\text{Weight of solution}} \times 100$$

$$\text{Weight/volume percent (\% w/v)} = \frac{\text{Weight of solute (g)}}{\text{Volume of solution (mL)}} \times 100$$

$$\text{Milligram percent (mg\%)} = \frac{\text{Weight of solute (mg)}}{100 \text{ mL of solution}} \times 100$$

$$\text{Parts per million (ppm)} = \frac{\text{Weight of solute}}{\text{Weight of solution}} \times 10^6$$

Parts per million (for gases)

$$\text{ppm} = \frac{(\text{mg/m}^3)(R)}{\text{Molecular weight}}$$

where $R = 24.5$ at 25°C .

$$(\text{volume}_C)(\text{concentration}_C) = (\text{volume}_D)(\text{concentration}_D)$$

where

C is the concentrated solution

D is the dilute solution

The aforementioned relationship is useful in preparing dilute solutions from concentrated solutions.

D.3 pH CALCULATIONS

$$\text{pH} = -\log[\text{H}^+] = \log \frac{1}{[\text{H}^+]}$$

$$\text{pH} = \text{pK}_a + \log \frac{[\text{A}^-]}{[\text{HA}]}$$

where $\text{HA} \leftrightarrow \text{H}^+ + \text{A}^-$

(weak acid) (conjugate base)

$\text{pK}_a = -\log K_a$

(equilibrium constant)

D.4 PROPERTIES OF COMMONLY USED SUBSTANCES (TABLES D.1 THROUGH D.3)

TABLE D.1

Strengths of Concentrated Solutions of Acids and Bases

Acid or Base	Specific Gravity	Percentage by Weight	Weight (g/L)	Approximate Molarity
Hydrochloric acid (HCl)	1.19	37.0	440	12.1
Sulfuric acid (H ₂ SO ₄)	1.84	96.0	1730	18.0
Nitric acid (HNO ₃)	1.42	70.0	990	15.7
Acetic acid (CH ₃ COOH)	1.06	99.5	1060	17.4
Ammonium hydroxide (NH ₄ OH)	0.880	29.0	250	15–17
Sodium hydroxide (saturated solution) (NaOH)	1.50–1.53	About 50	600–700	15–18
Potassium hydroxide (saturated solution) (KOH)	1.55	About 50	800	14.0

TABLE D.2

Physiological Solutions (g/L)

	NaCl	KCl	CaCl ₂	MgCl ₂	NaHCO ₃	NaH ₂ PO ₄	KH ₂ PO ₄	MgSO ₄	Glucose
Saline (mammal)	9.00	—	—	—	—	—	—	—	—
Ringer (mammal)	9.00	0.42	0.24	—	0.50	—	—	—	1.00
Ringer (by Cattell)	9.00	0.42	0.12	—	—	—	0.100	—	1.00
Ringer (by Dresel)	6.00	0.531	0.35	—	2.10	—	0.081	0.147	0.90
Ringer (by Evans)	—	0.42	0.12	0.200	—	—	—	—	1.00 ^{a,b}
Ringer (by Genell)	8.00	0.42	0.24	0.005	1.00	—	—	—	0.50
Ringer (by Moran)	7.00	0.42	0.24	0.200	2.10	—	—	—	1.80
Ringer–Dale (by Stewart)	9.00	0.42	2.015	0.003	0.50	—	—	—	0.50
Ringer–Locke (same as Locke's)	9.00	0.42	.024	—	0.15	—	—	—	1.75
Ringer–Locke (by Feldberg)	9.00	0.20	0.20	—	0.30	—	—	—	1.00
Ringer–Locke (by Gaddum)	9.00	0.42	0.06	—	0.50	—	—	—	0.50
Ringer–Locke (by Hukovic)	9.00	0.42	0.24	—	0.50	—	—	—	2.00
Locke's (by Burn)	9.00	0.42	0.24	0.005	0.50	—	—	—	0.50
Krebs–Henseleit	6.87	0.40	0.28	—	2.10	0.140	—	0.140	2.00
Krebs–Henseleit–Ringer	6.90	0.354	0.280	—	2.10	—	0.162	0.294	—
Krebs–Henseleit (by Furchgott)	6.90	0.354	0.282	—	2.10	—	0.162	0.294	1.80
Krebs (by Hukovic)	6.60	0.350	0.280	—	2.10	—	0.162	0.294	2.08
Beauvilain's	9.00	0.42	0.06	0.005	0.50	—	—	—	0.50
McEwan's	7.60	0.42	0.24	—	2.10	0.143	—	—	2.00 ^c
Tyrode (isolated gut)	8.00	0.20	0.20	0.100	1.00	0.050	—	—	1.00
Feigen's (isolated heart)	9.00	0.42	0.62	—	0.60	—	—	—	1.00

^a K₂SO₄ = 22.00.^b KHCO₃ = 3.60.^c Sucrose = 4.50.

TABLE D.3
Composition of a Typical Organ Perfusion Medium

	Component	Concentration
Electrolytes	NaCl	115 mM
	KCl	5.4 mM
	MgSO ₄	2.4 mM
	CaCl ₂	3.0 mM
Buffer	NaH ₂ PO ₄	1.5 mM
	NaHCO ₃	25 mM
Source of energy	Glucose	5 mM
	Glutamine	2 mM
Oncotic agent	Bovine serum albumin	60 g/L
Other additions	Alanine	2 mM
	Glutathione	2 mM

D.5 GLOSSARY OF TERMS ASSOCIATED WITH SOLUTIONS

Dispersion: A two-phase system that consists of finely divided particles distributed throughout a bulk substance. Examples include gas in liquid (foam), solid in gas (aerosol), gas in solid (foamed plastic), liquid in gas (fog), liquid in liquid (emulsion), solid in liquid (paint), and solid in solid (carbon black in rubber).

Emulsion: A system containing two or more immiscible liquids in which one is dispersed in the form of very small globules throughout the other.

Miscible: Capable of being mixed and remaining so after the mixing process ceases. Materials that do not mix at all are said to be immiscible (e.g., oil and water).

Mixture: A mutual incorporation of two or more substances without chemical union, the physical characteristics of each of the components being retained. The components may or may not be uniformly dispersed and can usually be separated by mechanical means. Mixtures can be broadly grouped into two classes: *mechanical mixtures*, which consist of a mixture of particles or masses distinguishable as such under a microscope or by other methods, and *physical mixtures*, which consist of a more intimate mixture of molecules such as with gases and many solutions.

Solubility: The ability or tendency of one substance to blend uniformly with another. Examples include solid in liquid, liquid in liquid, gas in liquid, and gas in gas. Usually, liquids and gases are said to be miscible in other liquids and gases rather than soluble.

Soluble: Capable of being dissolved.

Solution: A uniformly dispersed mixture at the molecular or ionic level of one or more substances (the solute) in one or more other substances (the solvent).

Suspension: The dispersion through a liquid of very small particles (solid, semisolid, or liquid) of a size large enough to be detected by optical means. If the particles are small enough to pass through filter membranes but still large enough to scatter light, they will generally remain dispersed indefinitely and the system is called a colloidal suspension.

REFERENCES

- Sax, N. I. and Lewis, R. J. Sr., *Hawley's Condensed Chemical Dictionary*, 11th edn., Van Nostrand Reinhold Company, New York, 1987.
- Segel, I. H., *Biochemical Calculations*, 2nd ed., John Wiley & Sons, New York, 1976.
- Skoog, D. A. and West, D. M., *Fundamentals of Analytical Chemistry*, 2nd edn., Holt, Rinehart and Winston, New York, 1969.
- Stedman's Medical Dictionary*, 22nd edn., Williams & Wilkins Company, Baltimore, MD, 1972.

Appendix E: Design and Performance of Toxicology Studies

E.1 ACCEPTABLE TOXICOLOGY STUDY REQUIREMENTS

Table E.1 lists the minimum requirements that must be met for a toxicology study to be acceptable under present-day standards.¹ Some of these are addressed in detail in the referenced Good Laboratory Practice (GLP) Standards.²⁻⁴ Generally, US GLP satisfy similar regulatory requirements of other countries. Additional acceptability requirements may be associated with specific study designs (e.g., neurotoxicity and reprotoxicity) and need to be addressed on a case-by-case basis.

E.2 ESTIMATED COSTS AND TEST SAMPLE REQUIREMENTS FOR STANDARD TOXICOLOGY TESTS

Tables E.2 through E.4 provide estimated costs and test sample requirements for standard toxicology tests. The costs indicated are based on year 2012 estimates. For later years, add for inflation at a minimum. Test sample requirements, especially for longer-term studies by inhalation, can vary considerably and depend on the toxic potency of the material and the physical nature of the test substance (for inhalation studies). For example, an inhalation study of a minimally toxic dust will require orders of magnitude more test material than that of an equivalent study of a highly toxic gas.

When comparing costs, attention should be paid to the design of the study being offered. The cost of mammalian studies, particularly acute studies, can be influenced by several factors including the use of vehicle control groups, degree of gross and microscopic pathology required, use of positive controls, requirements for dedicated animal rooms, need for dose range-finding studies, type and number of

observations required, cost of animals, and the degree of statistical analyses and report format required. Analytical method development can add considerable costs to toxicology studies. The requirement for a flow-through design can increase the costs of aquatic studies. Factors affecting the cost of genetic studies include the number of bacterial strains and the method of exposure to liver enzymes utilized, the number of harvest times, the type and degree of end point analyses performed, and the need for range-finding studies and confirmatory tests.

Test costs do not necessarily correlate with quality. The most expensive labs may not always represent the best choice. Conversely, a lab should not be ruled out of consideration on the basis of low prices.

E.3 CONTRACT LABORATORIES

Table E.5 provides a directory of commercial contract research laboratories (CROs) that offer mammalian toxicology studies and, in some cases, genetic and/or aquatic toxicology studies. Detailed information about staff, facilities, and specific tests offered can be found on the organizations' website or by contacting the laboratories. Directories of laboratories providing ancillary testing related to toxicology such as physiochemical analysis, microbiological screening, and environmental fate analysis are published elsewhere and are beyond the scope of this book.

The information provided in this directory was reasonably accurate at the time of its preparation. This list includes CROs that have exhibited at recent Society of Toxicology (SOT) meetings or are otherwise known to the authors. Inclusion in this directory does not represent an endorsement of the laboratory; likewise, absence from this list does not indicate a lack of endorsement.

TABLE E.1**Minimum Requirements for Acceptable Toxicology Study**

1. The study should be conducted at a laboratory recognized by accreditation and/or reputation as having the scientific capability, expertise, and experience to conduct the study of interest.
 2. The study should be conducted according to GLP.
 3. The objectives and design of the study should be specified in a study-specific protocol approved by the study director and the sponsor (if applicable).
 4. The chemical nature of the tested material should be precisely defined and documented including chemical identity, stability, and degree of purity with any impurities clearly defined.
 5. The specificity of any methodology used should be adequate for the degree of detection of the end points to be evaluated. Such methods must be validated. Positive controls and standards should be used as necessary.
 6. The number of test and control animals should be sufficient to allow the detection of biological variability in response to exposure, to allow trends to be appreciated, and to be sufficient for statistical analyses.
 7. Ideally, doses or exposure levels should be sufficient to detect toxicity, define thresholds, and establish no-effect levels.
 8. Statistical procedures used should be appropriate for the type of data analyzed.
 9. The study should be reported in a clear and unambiguous manner with all necessary detail to allow the reader to understand the study design, interpret the results, and draw conclusions. All deviations from the protocol that have occurred should be clearly stated and the potential impact on the study assessed.
 10. The report should be signed by the study director to indicate agreement with the results and conclusions. In addition, the study director should sign the GLP compliance statement that states whether the study was conducted in full GLP compliance and if not, the areas of noncompliance. The report should also contain the signed quality assurance statement providing dates of inspection and reporting to management.
-

TABLE E.2
Mammalian Toxicology Studies: Cost and Material Requirements

Study Type	Typical Costs ^d	Estimated Material Requirements ^e
Acute Oral Toxicity in Rats, Limit Test	\$4,000	25 g
Acute Oral Toxicity in Rats, LD ₅₀ (four levels)	\$14,000	50 g
Acute Dermal Toxicity in Rabbits, Limit Test	\$5,000	50 g
Acute Dermal Toxicity in Rabbits, LD ₅₀ (four levels)	\$16,000	100 g
Acute Whole-Body Inhalation Toxicity in Rats, Limit Test	\$20,000	100–5,000 g
Acute Whole-Body Inhalation Toxicity in Rats, LC ₅₀	\$50,000	500–50,000 g
Primary Eye Irritation in Rabbits ^a	\$3,800	7.5 g
Primary Skin Irritation in Rabbits ^a	\$3,800	7.5 g
Dermal Sensitization in G. Pigs, Maximization ^b	\$12,000	40–80 g
Dermal Sensitization in G. Pigs, Buehler Type ^{b,c}	\$10,500	40–80 g
1-Month Oral Toxicity in Rats, Gavage	\$200,000	250 g
1-Month Whole-Body Inhalation Toxicity in Rats	\$325,000	1–200 kg
1-Month Intravenous Toxicity in Rats	\$240,000	20 g
1-Month Dermal Toxicity in Rats	\$220,000	250 g
1-Month Oral Toxicity in Dogs, Capsule	\$290,000	2.5 kg
1-Month Intravenous Toxicity in Dogs	\$300,000	200 g
3-Month Oral Toxicity in Rats, Gavage	\$325,000	750 g
3-Month Dietary Toxicity Study in Rats	\$300,000	800 g
3-Month Whole-Body Inhalation Toxicity in Rat	\$550,000	3–600 kg
3-Month Dermal Toxicity in Rats	\$350,000	750 g
3-Month Oral Toxicity in Dogs, Capsule	\$350,000	8 kg
3-Month Dietary Toxicity Study in Dogs	\$375,000	10 kg
6-Month Oral Toxicity in Rats, Gavage	\$500,000	1 kg
9-Month Oral Toxicity in Dogs, Capsule	\$800,000	15 kg
24-Month Oncogenicity in Mice, Gavage	\$1,750,000	1.5 kg
24-Month Oncogenicity in Rats, Gavage	\$1,750,000	18 kg
24-Month Whole-Body Inhalation in Rats	\$3,750,000	20–4,000 kg
General Fertility and Reproductive Performance (Segment I) in Rats	\$200,000	750 g
Range Finding Developmental Toxicity Study in Rats	\$50,000	90 g
Developmental Toxicity Study (Segment II) in Rats	\$140,000	200 g
Range Finding Developmental Toxicity Study in Rabbits	\$66,000	400 g
Developmental Toxicity Study (Segment II) in Rabbits	\$180,000	1 kg
Perinatal and Postnatal Study (Segment III) in Rats	\$325,000	750 g
Two-Generation Reproduction Study in Rats	\$450,000	8 kg

^a Additional cost if extended observation periods are required.

^b Additional cost for positive control.

^c Number of induction times may vary.

^d Based on 2012 costs.

^e Material requirements, especially for longer-term studies by whole body inhalation, can vary considerably depending on toxic potency of the chemical substance as well as its physical properties (e.g., dust vs. gas for inhalation studies).

TABLE E.3
Genetic Toxicology Studies: Cost and Material Requirements

Study Type	Typical Costs ^a	Estimated Material Requirements
Ames Assay	\$7,000	1.5–5 g
Mouse Lymphoma Assay	\$33,000	5 g
<i>In Vitro</i> Chromosome Aberrations (CHO)	\$31,000	5 g
<i>In Vitro</i> Chromosome Aberrations (Human Lymphocytes)	\$38,000	5 g
<i>In Vitro</i> Chromosome Aberrations (Rat Lymphocytes)	\$36,000	5 g
<i>In Vivo</i> Chromosome Aberrations (Mouse Bone Marrow)	\$40,000	10–15 g
<i>In Vivo</i> Chromosome Aberrations (Rat Bone Marrow)	\$41,000	60 g
<i>In Vitro</i> Unscheduled DNA Synthesis (UDS)	\$19,000	5 g
<i>In Vivo/In Vitro</i> UDS	\$50,000	25–50 g
<i>In Vitro</i> SHE Cell Transformation (Syrian Hamster Embryo Cells)	\$26,000	25–30 g
Mouse Micronucleus	\$14,000 (males)	
	\$27,000 (males and females)	10–15 g
<i>In Vitro</i> Sister Chromatid Exchange (SCE)	\$16,000	5 g
<i>In Vivo</i> SCE (Mouse)	\$36,000	15 g
<i>In Vivo</i> Rodent Comet Assay (Rat)	\$28,000	30 g
<i>In Vivo</i> Rodent Comet and Micronucleus Combination Assay (Rat)	\$48,000	30 g

^a Based on 2012 costs.

TABLE E.4
Aquatic/Ecotoxicology Studies: Cost and Material Requirements

Study Type	Typical Costs ^a	Estimated Material Requirements
Fish Static Acute (96 h)	\$4,500	10 g
Fish Early Life Stage	\$44,000	350 g
Daphnid Static Acute (48 h)	\$4,200	5 g
Daphnid 21-Day Chronic Reproduction	\$20,000	100 g
Algal Static Acute (96 h)	\$5,000	5 g
Algal Static 14-Day	\$13,000	5 g
Fish Bioconcentration	\$78,000	200 g
Earthworm (48 h—Filter Paper)	\$5,000	5 g
Earthworm (14-Day—Soil)	\$6,500	30 g

^a Based on 2012 costs.

TABLE E.5
Directory of Contract Toxicology Laboratories

Absorption Systems

436 Creamery Way, Suite 600
Exton, PA 19341
United States
Phone: 610-280-7300
www.absorption.com

Accelera S.r.l.

Viale Pasteur 10
Nerviano (Milano), 20014
Italy
www.accelera.org

Advinus Therapeutics Limited

21 & 22, Phase II Peenya Industrial Area
Bangalore, 500058
India
Phone: 91-9008490277
www.advinus.com/

Aptuit LLC

Two Greenwich Office Park
Greenwich, CT 06831
United States
Phone: 855-506-6360
www.apuit.com

Avanza Laboratories, LLC

11 Firstfield Road, Suite 11-B
Gaithersburg, MD 20878
United States
Phone: 240-364-6360
www.avanzalaboratories.com

BASi (Bioanalytical Systems, Inc.)

10424 Middle Mt. Vernon Road
Mt. Vernon, IN 47620
United States
Phone: 812-985-3400
www.basinc.com

Battelle

505 King Avenue
Columbus, OH 43201
United States
Phone: 800-201-2011
www.battelle.org

Bioagri Pharma

Rodovia SP 127, Km 24
Piracicaba, SP, 13400-412
Brazil
Phone: 55-19-3429-7739
<http://www.bioagri.com.br>

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Biological Test Center (BTC)

2525 McGaw Avenue
Irvine, CA 92614
United States
Phone: 949-660-3185
www.biologicaltestcenter.com

BioReliance Corporation

14920 Broschart Road
Rockville, MD 20855
United States
Phone: 301-738-1000
www.bioreliance.com

Biosafety Research Center, Foods, Drugs and Pesticides (BSRC)

582-2, Shioshinden
Iwata, Shizuoka,
Japan
Phone: 81-538-58-1266
www.anpyo.or.jp

BioTox Sciences

PO Box 910418
San Diego, CA 92191
United States
Phone: 858-605-5882
www.biotoxsciences.com

Biotrial

7-9 rue Jean-Louis Bertrand
Rennes, 18940
France
Phone: +33 0 2 99 599 191
www.biotrial.com

Burleson Research Technologies

120 First Flight Lane
Morrisville, NC
United States
Phone: 919-719-2500
www.brt-labs.com

Calvert Laboratories, Inc.

130 Discovery Drive
Scott Township, PA 18447
United States
Phone: 570-586-2411
www.calvertlabs.com

CARE Research LLC/Colorado Histo-Prep

6200 East County Road 56
Fort Collins, CO 80524
United States
Phone: 970-493-2660
www.carereseearchllc.com

(continued)

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

CBSET, Inc.

500 Shire Way
 Lexington, MA 02421
 United States
 Phone: 781-541-5555
www.cbset.org

Charles River

251 Ballardvale Street
 Wilmington, MA 01887
 United States
 Phone: 781-222-6187
www.criver.com

CiToxLAB

445 Armand-Frappier Boulevard
 Laval, QC H7V 4B3
 Canada
 Phone: 450-973-2240
www.citoxlab.com/

Comparative Biosciences Inc. (CBI)

786 Lucerne Drive
 Sunnyvale, CA 94086
 United States
 Phone: 408-738-9260
www.compbio.com

CorDynamics

2242 W. Harrison St Suite 108
 Chicago, IL 60612
 United States
 Phone: 312-421-8876
www.cordynamics.com

Covance Inc.

3301 Kinsman Blvd.
 Madison, WI 53704
 United States
 Phone: 608-395-3621
www.covance.com

CXR Biosciences Limited

2 James Lindsay Place, Dundee Technopole
 Dundee, Tayside DD1 5JJ
 United Kingdom
 Phone: +44 1382 432163
www.cxbiosciences.com

Experimur

4045 S. Morgan Street
 Chicago, IL 60609
 United States
 Phone: 773-254-2700-232
www.experimur.com

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Fraunhofer ITEM

Nikolai-Fuchs Strasse 1
 Hannover, 30625
 Germany
 Phone: 49-511-5350-462
www.item.fraunhofer.de

Frontier BioSciences, Inc.

20251 Century Blvd. Suite 325
 Germantown, MD 20874
 United States
 Phone: 301-515-5560
www.frontierbsi.com/

Harlan Laboratories, Inc.

8520 Allison Pointe Boulevard Suite 400
 Indianapolis, IN 46250
 United States
 Phone: 317-806-6080
www.harlan.com

Huntingdon Life Sciences

P.O. Box 2360, Mettlers Road
 East Millstone, NJ 08875
 United States
 Phone: 732-873-2550
www.huntingdon.com

IIT Research Institute (IITRI)

10 W 35th Street
 Chicago, IL 60616
 United States
 Phone: 312-567-4924
www.iitri.org/

Ina Research, Inc.

2148-188 Nishiminowa Nagano-ken
 Ina-shi, Nagano-ken 399-4501
 Japan
 Phone: 81-265-72-6616
www.ina-research.co.jp/

Institute for In Vitro Sciences

30 West Watkins Mill Road, Suite 100
 Gaithersburg, MD 20878
 United States
 Phone: 301-947-6523
www.iivs.org

Institute of Industrial Organic Chemistry Branch Pszczyna

Doświadczalna 27
 Pszczyna, 43-200
 Poland
 Phone: 48322103081
www.ipo-pszczyna.pl

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Isis Services

1031 Bing St
 San Carlos, CA 94070
 United States
 Phone: 510-704-0140
 www.isis-services.com

INTOX Pvt. Ltd.

375, Urawade, Tal. Mulshi
 Dist. Pune, Maharashtra 411042
 India
 Phone: +912066548700
 www.intoxlab.com

ITR Laboratories Canada, Inc.

19601 Clark Graham Boulevard
 Baie D'Urfe (Montreal), QC H9X 3T1
 Canada
 Phone: 514-457-8527
 www.itrlab.com

Jai Research Foundation (JRF America)

2650 Eisenhower Avenue Suite C
 Audubon, PA 19403
 United States
 Phone: 484-681-0523
 www.jrfamerica.com

Joinn Laboratories

20271 Goldenrod Ln, Suite 2020
 Germantown, MD 20876
 United States
 Phone: 301-540-5988
 www.joinnlaboratories.com

Korea Institute of Toxicology (KIT)

141 Gajeong-ro, Yuseong-gu P.O Box 123
 Daejeon, 305-600
 Korea (South)
 Phone: 82.42.610.8204
 www.kitox.re.kr/eng

Kunming Biomed International (KBI)

Boda Rd, Yuhua Area, Cheng-Gong New Town
 Kunming
 China
 Phone: 868715952828
 www.kbimed.com

Lovelace Respiratory Research Institute (LRRI)

2425 Ridgecrest Drive SE
 Albuquerque, NM 87108
 United States
 Phone: 505-348-9456
 www.lrrri.org

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Maccine Pte Ltd.

10 Science Park Road, The Alpha #01-05 Singapore Science Park II
 Singapore, 117684
 Singapore
 Phone: 202-558-0262
 www.maccine.com

MB Research Laboratories

1765 Wentz Road
 Spinnerstown, PA 18968
 United States
 Phone: 215-536-4110
 www.mbresearch.com

Medicilon Preclinical Research (Shanghai) LLC

585 Chuanda Road, Chuansha Economic Park
 Shanghai
 China
 Phone: 86-21-58591500
 www.medicilon.com

MPI Research

54943 N. Main Street
 Mattawan, MI 49071
 United States
 Phone: 269-668-3336
 www.mpiresearch.com

NAMSA

6750 Wales Road
 Northwood, OH 43619
 United States
 Phone: 419-662-4871
 Fax: 419-662-4802
 www.namsa.com

National Research Center for New Drug Safety Evaluation (Shenyang)

43508 Castlewood
 Novi, MI 48375
 United States
 Phone: 248-270-3123
 www.toxisafe.com

National Shanghai Center for New Drug Safety Evaluation & Research

199 Guoshoujing Road
 Shanghai, 201203
 China
 Phone: 86-21-50800333
 www.ncdser.com/

NIA Life Sciences, Inc.

28100 Ashley Circle, Suite 102
 Libertyville, IL 60048
 United States
 Phone: 847-996-1851
 www.nialifesciences.com

(continued)

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Nucro-Technics Incorporated

2000 Ellesmere Road Unit #16
 Scarborough, ON M1H 2W4
 Canada
 Phone: 416-438-6727
www.nucro-technics.com

Pacific BioLabs

551 Linus Pauling Drive
 Hercules, CA 94547
 United States
 Phone: 510-964-9000
www.pacificbiolabs.com

Pharmaron (formerly Bridge Laboratories)

6 Venture Ste. 250
 Irvine, CA 92618
 United States
 Phone: 949-788-0586
www.pharmaron.com

Pre-Clinical Research Services, Inc. (PCRS)

1512 Webster Ct.
 Fort Collins, CO 80524
 United States
 Phone: 970-232-1122
www.preclinicalresearch.com

Porsolt

9 bis rue Henri Martin
 BOULOGNE-BILLANCOURT, 92100
 France, Metropolitan
 Phone: 33146109990
www.porsolt.com

QPS LLC

Three Innovation Way, Suite 240
 Newark, DE 19711
 United States
 Phone: 302-690-4962
www.qps-usa.com

Ricerca Biosciences

7528 Auburn Road
 Concord, OH 44077
 United States
 Phone: 610-213-5408
www.ricerca.com

RTC, Research Toxicology Centre S.p.A.

Via Tito Speri, 12
 Pomezia (Rome), 40
 Italy
 Phone: (39) 06-910-95 263
www.rtc.it

RTI International

3040 East Cornwallis Road Bldg. 9 Rm. 102
 Research Triangle Park, NC 27709
 United States
 Phone: 919-541-6850
www.rti.org

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Sequani Limited

Bromyard Road
 Ledbury, Herefordshire HR8 1LH
 United Kingdom
 Phone: 00441531 634121
www.sequani.com

Seventh Wave

743 Spirit 40 Park Drive Suite 209
 Chesterfield, MO 63005
 United States
 Phone: 636-519-4885
www.7thwavelabs.com/

Sinclair Research Center LLC

P.O. Box 658
 Columbia, MO 65205
 United States
 Phone: 585-742-2573
www.sinclairresearch.com

Sitek Research Laboratories

15235 Shady Grove Road suite 303
 Rockville, MD 20850
 United States
 Phone: 301-926-4900
www.siteklabs.com

Smithers

790 Main Street
 Wareham, MA 02571
 United States
 Phone: 508-295-2550
www.smithers.com

SNBL USA, Ltd.

6605 Merrill Creek Parkway
 Everett, WA 98203
 United States
 Phone: 425-322-1950
www.snblusa.com

SoBran Inc.

2677 Prosperity Avenue, Suite 200
 Fairfax, VA 22031
 United States
 Phone: 205-514-9778
www.sobran-inc.com

Southern Research

2000 9th Avenue South
 Birmingham, AL 35205
 United States
 Phone: 205-581-2203
www.southernresearch.org

Spring Valley Laboratories, Inc. (SVL)

P.O. Box 242
 Woodbine, MD 21797
 United States
 Phone: 410-795-2222
www.svlab.com

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

SRI International

333 Ravenswood Avenue
 Menlo Park, CA 94025
 United States
 Phone: 650-859-3000
www.sri.com/about/organization/biosciences

Stillmeadow Inc.

12852 Park One Drive
 Sugar Land, TX 77498
 United States
 Phone: 281-240-8828
www.stillmeadow.com

Suven Life Sciences Limited

Serene Chambers, Road - 5, Avenue - 7 Banjara Hills
 Hyderabad, 500034
 India
 Phone: +91-40-23556038
www.suven.com

Syngene International Limited

Plot No. 2&3, Bommasandra IV Phase, Jigani Link Road
 Bangalore, KARNATAKA 560099
 India
 Phone: +9198805 99557/+91 80280 83161
www.syngeneintl.com

Toxicology Research Laboratory (TRL)

808 South Wood Street room 1306
 Chicago, IL 60612
 United States
 Phone: 312-996-9185
www.uic.edu/labs/tox/trlt

TNO

Utrechtseweg 48 P.O. Box 844
 Zeist, Utrecht 3700 AV
 Netherlands
 Phone: +31 88 866 16 28
www.triskelion.nl

Toxi-Coop Toxicological Research Center

Magyar Jakobinusok tere 4/B
 Budapest, H-1122
 Hungary
 Phone: +36308462664
www.trc.hu/

Toxikon Corporation

15 Wiggins Avenue
 Bedford, MA 01730
 United States
 Phone: 781-275-3330
www.toxikon.com

TABLE E.5 (continued)
Directory of Contract Toxicology Laboratories

Triangle Research Labs, LLC

6 Davis Drive P.O. Box 14453
 Research Triangle Park, NC 27709
 United States
 Phone: 919-549-3593
www.gigacyte.com

Vanta Bioscience LC

5550 Wild Rose Lane
 West Des Moines, IA 50266
 United States
 Phone: 732-812-4222
www.vantabio.com

Vivo Bio Tech Limited

A1, III Floor, Surabhi Plaza Vikramপুরi
 Secunderabad, Andhra Pradesh 500009
 India
 Phone: +91-40-27890662
www.vivobio.com

WestChina-Frontier PharmaTech Co., Ltd.

28 Gaopeng Ave., Hi-tech Development Zone
 Chengdu, Sichuan 610041
 China
 Phone: +86-186-8163-6996
www.frontierbsi.com

WIL Research

1407 George Road
 Ashland, OH 44805
 United States
 Phone: 419-289-8700
www.wilresearch.com

Wildlife International, Ltd.

8598 Commerce Drive
 Easton, MD 21601
 United States
 Phone: 410-822-8600
www.wildlifeinternational.com

WuXi AppTec

2540 Executive Drive
 St. Paul, MN 55120
 United States
 Phone: 651-675-2000
www.wuxiapptec.com

Xenometrics/Product Safety Labs

2394 Route 130 Suite E
 Dayton, NJ 08810
 United States
 Phone: 732-438-5100
www.xenometricsllc.com

REFERENCES

- Ballantyne, B. B. and Sullivan, J. B., Basic principles of toxicology, in *Hazardous Materials Toxicology: Clinical Principles of Environmental Health*, Sullivan, J. B., and Krieger, G. R., Eds., Williams and Wilkins, Baltimore, MD, 1992, chap. 2.
- Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Regulations; Final Rule, Code of Federal Regulations (CFR) 40, Part 160, United States Environmental Protection Agency, 1989.
- Food and Drug Administration; Good Laboratory Practice Regulations; Final Rule, Code of Federal Regulations (CFR) 21, Part 58, 1991 (revision), United States Department of Health and Human Services.
- Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards; Final Rule. Code of Federal Regulations (CFR) 40, Part 792, United States Environmental Protection Agency, 1989.

Appendix F: Organizations and Agencies Associated with Toxicology and/or Toxicological Issues

Academy of Toxicological Sciences (ATS)

1821 Michael Faraday Drive, Suite 300
Reston, VA 20190
Horsham, PA 19044
(703) 438-3103
www.acadtox.org

**Agency for Toxic Substances and Disease Registry
(ATSDR)**

4770 Buford Hwy. NE
Atlanta, GA 30341
1-800-232-4636
www.atsdr.cdc.gov

American Academy of Clinical Toxicology

6728 Old McLean Village Drive
McClean, VA 22101
(703) 556-9222
www.clintox.org

**American Academy of Veterinary and Comparative
Toxicology (AAVCT)**

P.O. Box 6050
Fargo, ND 58108
(701) 231-7529
www.aavct.org

**American Association of Poison Control Centers
(AAPCC)**

515 King Street, Suite 510
Alexandria, VA 22314
(703) 894-1858
www.aapcc.org

**American (Board) College of Medical Toxicology
(ACMT)**

10645 N. Tatum Blvd., Suite 200-111
Phoenix, AZ 85028
(623) 533-6340
www.acmt.net

American Board of Toxicology (ABT)

PO Box 97786
Raleigh, NC 27624
(919) 841-5022
www.abtox.org

American Chemical Society (ACS)

1155 16th Street, NW
Washington, DC 20036
(202) 872-4600
www.acs.org

American Chemistry Council (ACC)

700 Second Street, NE
Washington, DC, 20002
(202) 249-7000
www.americanchemistry.com

American College of Toxicology (ACT)

1821 Michael Faraday Drive
Suite 300 Reston, VA 20190
(703) 547-0875
www.actox.org

**American Conference of Governmental Industrial
Hygienists (ACGIH)**

1330 Kemper Meadow Drive
Cincinnati, OH 45240
(513) 742-6163
www.acgih.org

American Industrial Hygiene Association (AIHA)

3141 Fairview Park Drive, Suite 777
Falls Church, VA 22042
(703) 207-3561
www.aiha.org

**American Society for Pharmacology and
Experimental Therapeutics (ASPET)**

9650 Rockville Pike
Bethesda, MD 20814
(301) 634-7060
www.aspet.org

Board on Environmental Studies and Toxicology

The National Academies
500 Fifth Street, NW
Washington, DC 2001
(202) 334-3600
www.dels.nas.edu/best

Centers for Disease Control and Prevention

1600 Clifton Road, NE
Atlanta, GA 30333
1-800-232-4636
www.cdc.gov

Consumer Product Safety Commission (CPSC)

4330 East-West Highway
Bethesda, MD 20814
(301) 504-7923
www.cpsc.org

Department of Agriculture (USDA)

1400 Independence Avenue, SW
Washington, DC 20250
(202) 720-2791
www.usda.gov

Department of Energy (DOE)

1000 Independence Avenue SW
Washington, DC 20585
(202) 586-5000
www.energy.gov

Department of the Interior Fish and Wildlife Service

Washington, DC 20240
1-800-344-9453
www.fws.gov

Department of Transportation (DOT) Office of Hazardous Material Technology

Pipeline and Hazardous Materials Safety Administration
1200 New Jersey Avenue, SE
East Bldg., 2nd Floor
Washington, DC 20590
(202) 366-4488
www.phmsa.dot.gov/hazmat/about

Environmental Defense Fund (EDF)

1875 Connecticut Avenue, NW, Suite 600
Washington, DC 20009
1-800-684-3322
www.edf.org

Environmental Law Institute (ELI)

2000 L Street, NW, Suite 620
Washington, DC 20038
(202) 939-3800
www.eli.org

Environmental Mutagen Society (EMS)

1821 Michael Faraday Drive, Suite 300
Reston, VA 20190
(703) 438-8220
www.ems-us.org

Environmental Protection Agency (EPA)

Ariel Rios Building
1200 Pennsylvania Avenue, NW
Washington, DC 20460
(202) 272-0167
www.epa.gov

EPA**Region 1 (CT, MA, ME, NH, RI, VT)**

5 Post Office Square, Suite 100
Boston, MA 02109
(617) 918-1111

EPA**Region 2 (NJ, NY, PR, VI)**

290 Broadway
New York, NY 10007-1866
(212) 637-3000

EPA**Region 3 (DC, DE, MD, PA, VA, WV)**

1650 Arch Street
Philadelphia, PA 19103-2029
(215) 814-5000

EPA**Region 4 (AL, FL, GA, KY, MS, NC, SC, TN)**

Atlanta Federal Center
61 Forsyth Street, SW
Atlanta, GA 30303-3104
(404) 562-9900

EPA**Region 5 (IL, IN, MI, MN, OH, WI)**

77 West Jackson Boulevard
Chicago, IL 60604-3507
(312) 353-2000

EPA**Region 6 (AR, LA, NM, OK, TX)**

Fountain Place, 12th Floor, Suite 1200
1445 Ross Avenue
Dallas, TX 75202-2733
(214) 665-2200

EPA**Region 7 (IA, KS, MO, NE)**

901 North 5th Street
Kansas City, KS 66101
(913) 551-7003

EPA**Region 8 (CO, MT, ND, SD, UT, WY)**

1595 Wynkoop Street
Denver, CO 80202
(303) 312-6312

EPA**Region 9 (AZ, CA, HI, NV)**

75 Hawthorne Street
San Francisco, CA 94105
(415) 947-8000

EPA**Region 10 (AK, ID, OR, WA)**

1200 Sixth Avenue, Suite 900
Seattle, WA 98101
(206) 553-1200

European Society of Toxicology (EUROTOX)

www.eurotox.com

Food and Drug Administration (FDA)

10903 New Hampshire Avenue
Silver Springs, MD 20993
1-888- 463-6332
(301) 827-0372 Center for Biologics Evaluation and Research (CBER)
(301) 796-5400 Center for Devices and Radiological Health (CDRH)
(301) 796-5400 Center for Drug Evaluation and Research (CDER)
(240) 402-1600 Center for Food Safety and Applied Nutrition (CFSAN)
(240) 276-9000 Center for Veterinary Medicine (CVM)
(870) 543-7130 National Center for Toxicological Research (NCTR)
(301)-827-7130 Office of Regulatory Affairs

Genetic Toxicology Association (GTA)

www.gta-us.org

**The Hamner Institutes for Health Research
(Formerly Chemical Industry Institute of
Toxicology)**

6 Davis Drive
P.O. Box 12137
Research Triangle Park, NC 27709
(919) 558-1200
www.thehamner.org

International Life Sciences Institute (ILSI)

1156 Fifteenth Street, NW Suite 200
Washington, DC 20005
(202) 659-0074
www.ilsa.org

**International Society of Regulatory Toxicology and
Pharmacology (ISRTP)**

6546 Belleview Drive
Columbia, MD 21046
(410) 992-9083
www.isrtp.org

Inhalation Toxicology Research Institute (ITRI)

US Department of Energy
P.O. Box 5890
Albuquerque, NM 87185
(505) 845-1037
www.em.doe.gov/bemr/bemrsites/itri.aspx

International Union of Toxicology (IUTOX)

1821 Michael Faraday Drive, Suite 300
Reston, VA 20190
703-438-3103
www.iutox.org
(Note: IUTOX has over 60 international toxicology societies as members. Contacts for member societies can be obtained from the IUTOX web site.)

National Cancer Institute (NCI)

6116 Executive Boulevard, Suite 300
Bethesda, MD 20892
1-800-422-6237
www.cancer.gov

National Center for Toxicological Research (NCTR)

USFDA
3900 NCTR Road
Jefferson, AR 72079
870-543-7000
www.fda.gov (search "NCTR")

National Eye Institute (NEI)

9000 Rockville Pike
Bethesda, MD 20892
(301) 496-5248
www.nei.nih.gov

National Heart, Lung, and Blood Institute

NHLBI Health Information Center
P.O. Box 30105
Bethesda, MD 20824
(301) 592-8573
www.nhlbi.nih.gov

**National Institute of Environmental Health Sciences
(NIEHS)**

111 Alexander Drive
P.O. Box 12233
Research Triangle Park, NC 27709
(919) 592-8573
www.niehs.nih.gov

National Institutes of Health

9000 Rockville Pike
Bethesda, MD 20892
(301) 496-4000
www.nih.gov

National Institute for Occupational Safety and Health (NIOSH)

1600 Clifton Road
Atlanta, GA 30333
1-800-232-6348
outside United States (513) 533-8328
www.cdc.gov/niosh

National Library of Medicine (NLM)

Toxicology Information Program (TIP)
8600 Rockville Pike
Bethesda, MD 20894
(888) 346-3656
outside United States (301) 594-5983
www.nlm.nih.gov

National Toxicology Program

Public Information Office
P.O. Box 12233, MDK2-05
Research Triangle Park, NC 27709
(919) 541-3419
www.ntp.niehs.nih.gov

Occupational Safety and Health Administration (OSHA)

200 Constitution Avenue, NW
Washington, DC 20210
1-800-321-6742
www.osha.gov

Oak Ridge National laboratory

P.O. Box 2008
One Bethel Valley Road
Oak Ridge, TN 37831
(865) 576-7658
www.ornl.gov

Organisation for Economic Co-operation and Development (OECD)

2 rue Andre Pascal
75775 Paris CEDEX 16, France
+33 1 45 24 82 00
www.oecd.org

Personal Care Products Council (Formerly Cosmetic Toiletry and Fragrance Association)

1101 17th Street, NW, Suite 300
Washington, DC 20036
(202) 331-1770
www.ctfa.org

Society of Environmental Toxicology and Chemistry (SETAC)

229 South Baylen Street
Pensacola, FL 32502
(850) 469-1500
www.setac.org

Society of Forensic Toxicologists (SOFT)

One MacDonald Center
1 N Macdonald Street, Suite 15
Mesa, AZ 85211
1-888-866-7638
www.soft-tox.org

Society of Toxicologic Pathology

1821 Michael Faraday Drive, Suite 300
Reston, VA 20190
(703) 438-7508
www.toxpath.org

Society of Toxicology (SOT)

1821 Michael Faraday Drive, Suite 300
Reston, VA 20190
(703) 438-3115
www.toxicology.org

Society of Chemical Manufacturers & Affiliates (SOCMA) (Formerly Synthetic Organic Chemical Manufacturers Association)

1850 M Street, NW, Suite 700
Washington, DC 20036
(202) 721-4100
www.socma.com

Teratology Society

1821 Michael Faraday Drive, Suite 300
Reston, VA 20190
(703) 438-3104
www.teratology.org

Toxicology Forum

1300 Eye Street, NW, Suite 1010 East
Washington, DC 20005
(202) 659-0030
www.toxforum.org

United Nations Environment Programme (UNEP)

United Nations Avenue, Gigri
P.O. Box 30552, 00100
Nairobi, Kenya
(254-20) 7621234
www.unep.org

World Health Organization

Avenue Appia 20
1211 Geneva 27, Switzerland
+41 22 791 21 11
www.who.int

Appendix G: Acronyms

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care (International)	ASTHO	Association of State and Territorial Health Officials
AADA	Abbreviated Antibiotic Drug Application	ASTM	Association of Standard Test Methods
AALAS	American Association for Laboratory Animal Science	AWA	Animal Welfare Act
AAPCO	Association of American Pesticide Control Officials	BAB	Biological Analysis Branch (re: BEAD of OPP)
ACB	Analytical Chemistry Branch (re: OPP)	BARQA	The British Association of Research Quality Assurance
ACC	American Chemistry Council	BDAT	Best demonstrated available technology
ACP	Associates of Clinical Pharmacology	BEAD	Biological and Economic Analysis Division (re: OPP)
ACS	American Chemical Society	CANDAs	Computer-assisted new drug applications
ACT	American College of Toxicology	CANADA	Computer-assisted new animal drug application
ACUP	Animal care and use procedure	CAP	Compliance audit program
ADE	Adverse drug experience/effect/event	CAPER	Computer-assisted preclinical electronic review
ADI	Acceptable daily intake (see also RfD)	CAPLA	Computer-assisted product license application (Re: Biologics)
ADME	Absorption distribution, metabolism, and excretion	CB	Communications branch (re: OPP)
ADR	Adverse drug reaction	CB I & II	Chemistry Branch I and II (re: OPP)
AE	Adverse experience/event	CBER	Center for Biologics Evaluation and Research (re: FDA)
AERS	Adverse Event Reporting System	CBI	Confidential business information
AHI	Animal Health Institute	CDC	Centers for Disease Control (see also USCDC)
ai/A	Active ingredient per acre	CDER	Center for Drug Evaluation and Research (re: FDA)
AI	Active ingredient	CDRH	Center for Devices and Radiological Health (re: FDA)
ALISS	“A-List” Inventory Support System (re: SRRD)	CEO	Council on Environmental Quality
ALJ	Administrative law judge	CERCLA	Comprehensive, Environmental, Response, Compensation, and Liability Act
ANADA	Abbreviated new animal drug application	CFD	Call for data
ANDA	Abbreviated new drug application	CFR	Code of Federal Regulations
ANSI	American National Standards Institute	CGMP	Current good manufacturing practices (see also GMP)
AOAC	Association of Official Analytical Chemists	CLIA	Clinical Laboratory Improvement Act
ARAR	Applicable or relevant and appropriate requirements (re: Superfund)	CMA	Chemical Manufacturers Association
APB	Antimicrobial Program Branch	CMC	Chemistry, Manufacturing, and Controls
ARB	Accelerated Reregistration Branch (re: SRRD of OPP)	CNAEL	Committee on National Accreditation for Environmental Laboratories
ARS	Agricultural Research Service (re: USDA)	CORT	Toxicology studies set: chronic feeding; oncogenicity; reproduction; teratology
ARTS	Accelerated Reregistration Tracking System (re: SRRD of OPP)		
ASAP	Administrative System Automations Project		
ASQC	American Society of Quality Control		
ASR	Analytical Summary Report		

CPDA	Chemical Producers and Distributors Association	ELA	Establishment license report (re: Biologics)
CPG	Compliance Policy Guide	ELGIN	Environmental Liaison Group International
CPGM	Compliance program guidance manuals (re: bioresearch monitoring program)	ELI	Environmental Law Institute
CPSC	Consumer Product Safety Commission	EMO	Experimental manufacturing order
CRA	Clinical research associate	EP	End-use product
CRADA	Cooperative research and development agreement	EPA	Environmental Protection Agency (see also USEPA)
CRF	Case report form	EPCRA	Emergency Planning and Community Right-to-Know Act
CRO	Contract research organization	EPRS	Establishment/product registration system
CRP	Child-resistant packaging	ESA	Entomological Society of America
CSA	Clinical & Scientific Affairs	EUP	Experimental use permit (re: EPA FIFRA)
CSF	Confidential Statement of Formula	FACTS	Field Accomplishments and Compliance Tracking System
CSMA	Chemical Specialties Manufacturers' Association	FDA	Food and Drug Administration (see also USFDA)
CSO	Consumer safety officer (re: FDA)	FDB	Field data book
CSRS	Cooperative State Research Service	FD&C	Federal Food, Drug, and Cosmetic Act (see also FFDCA, FDCL)
CTB	Certification and Training Branch (re: FOD of OPP)	FDCL	Food, Drug, and Cosmetic Law (see also FD&C, FFDCA)
CV	Curriculum vitae	FDLI	Food and Drug Law Institute
CVM	Center for Veterinary Medicine (re: FDA)	FFDCA	Federal Food, Drug, and Cosmetic Act (see also FD&C, FDCL)
CWA	Clean Water Act	FHB	Fungicide–Herbicide Branch (re: RD of OPP)
DAMOS	Drug Application Methodology with Optical Storage (re: EC)	FHSA	Federal Health and Safety Act
DCI	Data callin notice (re: RD or SRRD of OPP)	FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
DEA	Drug Enforcement Agency	FOD	Field Operations Division (re: OPP)
DEB	Dietary exposure branch (see also CB I and II)	FOI	Freedom of information (see also FOIA)
DFE	Design for the Environment	FOIA	Freedom of Information Act (see also FOI)
DI	Department of Interior (see also USDI)	FPLA	Fair Packaging and Labeling Act
DIA	Drug Information Association	FR	Federal Register
DIS	Drug information system	FRD	Field research director
DISLODG	Dislodgeable foliar residue (re: EPA)	FTC	Federal Trade Commission
DMF	Drug master file	FWS	Fish and Wildlife Service (see also USFWS)
DOE	Department of Energy (see also USDOE)	GALP	Good automated laboratory practices
DOT	Department of Transportation	GARPs	Good academic research practices (draft 1992)
DQOs	Data quality objectives (re: EPA work)	GAO	General Accounting Office
DRES	Dietary risk evaluation system (re: OPP)	GATT	General Agreement on Tariffs and Trade
EAB	Economic Analysis Branch (re: OPP)	GCPs	Good Clinical Practices
EC	Emulsifiable concentrate	GLPs	Good Laboratory Practices
EC	European Community (see also EEC)	GLPSs	Good Laboratory Practice Standards
EDF	Environmental Defense Fund	GMPs	Good manufacturing practices
EEB	Ecological Effects Branch (re: OPP)	GH ₂ O	Groundwater studies (re: EPA)
EEC	European Economic Community (see also EC)	GRAE	Generally recognized as effective
EFED	Environmental Fate and Effects Division (re: OPP)	GRAS	Generally recognized as safe
EFGWB	Environmental Fate and Groundwater Branch (re: OPP)		
EIR	Establishment inspection report		

HDT	Highest dose tested (re: EPA)	MOE	Margin of exposure
HED	Health Effects Division (re: OPP)	MOS	Margin of safety
HEI	Health Effects Institute	MOU	Memorandum of Understanding
HES	Health and environmental safety	MP	Manufacturing use product (re: OPP, see also MUP)
HHS	Health and human services	MPI	Maximum permitted intake
HIMA	Health Industry Manufacturers Association	MRID#	Master record identification number
HPB	Health Protection Branch (re: Canada)	MS	Master schedule
HPVSDS	High Production Volume Screening Info Data Set (re: OECD)	MSS	Master schedule sheet
HRS	Hazard Ranking System (re: Superfund)	MSDS	Material safety data sheet
IACUC	Institutional Animal Care and Use Committee	MTL	Master testing list
IB	Investigator's brochure	MUP	Manufacturing use product (re: OPP, see also MP)
ICH	International Conference on Harmonisation	MURS	Multiuser regulatory submission (International Multiagency Project)
ICR	Information collection request	NACA	National Agricultural Chemical Association
IDB	Investigational drug brochure	NADA	New animal drug application
IDE	Investigational device exemption	NADE	New animal drug evaluation, office of (re: FDACVM)
IG	Inspector general (see also OIG)	NAF	Notice of adverse findings
INAD	Investigational new animal drug	NAI	No action indicated
IND	Investigational new drug	NAICC	National Alliance of Independent Crop Consultants
IPM	Integrated pest management (re: OPP)	NARA	National Agrichemical Retailers Association
IR-4	Interregional Research Project #4 for Minor Crops (re: USDA)	NAS	National Academy of Sciences
IRB	Insecticide and Rodenticide Branch (re: RD of OPP)	NASDA	National Association of State Departments of Agriculture
IRB	Institutional review board	NCAMP	National Coalition Against the Misuse of Pesticides
IS	Information standards	NCE	New chemical entity
ISA	Information systems architecture	NCP	National contingency plan (re: Superfund)
ISB	Information Services Branch (re: PMSD of OPP)	NBS	National Bureau of Standards (now called NIST)
ISQA	International Society of Quality Assurance	NDA	New drug application
ITC	Interagency Testing Committee (re: TSCA)	NDS	New drug submission
LAC	Laboratory Accreditation Committee	NEIC	National Enforcement Investigation Center (re: USEPA)
LADD	Lifetime average daily dose (re: OPP)	NIEHS	National Institute of Environmental Health Sciences
LADD	Lowest acceptable daily dose (re: OPP)	NIH	National Institutes of Health
LC ₅₀	Lethal concentration for 50% of test population	NIOSH	National Institute for Occupational Safety and Health
LD ₅₀	Lethal dose for 50% of test population	NIST	National Institute of Standards and Technology (formerly NBS)
LDT	Lowest dose tested	NPCA	National Pest Control Association
LEL	Lowest effective level	NPDES	National Pollutant Discharge Elimination System
LRD	Laboratory research director	NPIRS	National Pesticide Information Retrieval System
LUIS	Label Use Information System (re: OPP)	NPL	National Priority List (re: Superfund)
MARSQA	Mid-Atlantic Region Society of Quality Assurance	NPTN	National Pesticide Telecommunications Network
MCA	Medicines Control Agency (United Kingdom equivalent to FDA)	NRDC	Natural Resources Defense Council
MCL	Maximum contaminant level		
MCLG	Maximum contaminant level goal		
MDDI	Medical devices, diagnostics and instrumentation		
MNVP	Medically necessary veterinary product (re: FDACVM)		

NTIS	National Technical Information Service	PMSD	Program management and support division (re: OPP)
OAI	Official action indicated	P&P guide	Policy and procedures guide (re: FDACVM)
OASIS	Operational and Administrative System for Import Support	PPIS	Pesticide Product Information Systems
ODW	Office of drinking water	PR#	Pesticide clearance request number
OECD	Organisation for Economic Co-operation and Development	PR notice	Pesticide registration notice
OECA	Office of Enforcement and Compliance Assurance	PRATS	Pesticide Registration Activity Tracking System
OES	Office of Endangered Species (re: FWS of DI)	PRCSQA	Pacific Regional Chapter of the Society of Quality Assurance
OGD	Office of Generic Drugs (re: FDA)	PRP	Potentially responsible party (re: Superfund)
OIG	Office of the Inspector General (see also IG)	PSA	Product safety assurance
OLTS	Online tracking system	QA	Quality assurance
OMB	Office of Management and Budget	QAAS	Quality assurance advisory subcommittee
OPM	Office of Personnel Management	QAO	Quality assurance officer
OPP	Office of Pesticide Programs (re: EPA)	QAP	Quality assurance project plan
OPPT	Office of Pollution Prevention and Toxics (formerly OTS; re: TSCA)	QAU	Quality assurance unit
OPPTS	Office of Prevention, Pesticides, and Toxic Substances	QC	Quality control
OREB	Occupational and Residential Exposure Branch (re: HED of OPP)	QMP	Quality management plan
OSB	Occupational Safety Branch (re: FOD of OPP)	QUA	Qualitative use assessment
OSHA	Occupational Safety and Health Administration	R&D	Research and development
OSW	Office of Solid Waste (re: EPA)	R&E	Research and experimental
OSWER	Office of Solid Waste and Emergency Response	RAC	Raw agricultural commodity (see also RACPC)
OTA	Office of Technology Assessment (re: Congress)	RAC	Risk Assessment Council
OTC	Over the counter	RACPC	Raw agricultural commodity processing (re: EPA)
OTS	Office of Toxic Substances (now called OPPT)	RAF	Risk Assessment Forum
OWPE	Office of Waste Programs Enforcement	RAPS	Regulatory affairs professional society
PAG	Pesticide Assessment Guidelines	RB	Reregistration Branch
PAI	Pure active ingredient	RCFs	Refractory ceramic fibers
PBA	Preliminary benefit analysis	RCRA	Resource Conservation and Recovery Act
PCO	Pest control operator	RD	Registration Division (re: OPP)
PDA	Parenteral Drug Association	RDRA	Remedial design/remedial action (re: Superfund)
PDMS	Pesticide document management system	RED	Reregistration Eligibility Document
PDR	Physician's Desk Reference	REI	Reentry interval (re: OPP)
PDUFA	Prescription Drug User Fee Act	RFC	Regional Field Coordinator
PES	Planning and evaluation staff	RfD	Reference dose
PHED	Pesticide handlers exposure database (re: EPA)	RFD	Recommended for development
PHI	Preharvest interval	RI/FS	Remedial investigation/feasibility study (re: Superfund)
PHI	Postharvest interval	RLC	Regional laboratory coordinator
PIMS	Pesticide Incident Monitoring System	RM	Review Manager (re: OPP)
PLA	Product license application (re: Biologics)	RM1	Risk management-1 (re: EPA)
PM	Product manager	RM2	Risk management-2 (re: EPA)
PMA	Pharmaceutical Manufacturers Association	RMEB	Resource Management and Evaluation Branch (re: PMSD of OPP)
PMN	Premanufacture notification (re: TSCA)	ROD	Record of decision (re: Superfund)
		RPAR	Rebuttable presumption against registration (re: OPP; see also SR)
		RRC	Regulatory Review Committee of the Society of Quality Assurance

RRD	Residue Research Director	TCLP	Toxicity characteristic leaching procedure, RCRA
RS	Registration standard (re: OPP)	TEP	Typical end-use product
RSB	Registration Support Branch	TFM	Testing facility management
RTECS	Registry of Toxic Effects of Chemical Substances	TGAI	Technical-grade active ingredient
RUP	Restricted use pesticide	TMRC	Theoretical maximum residue contribution (re: OPP)
SAB	Scientific advisory board (re: EPA)	TOSCA	Toxic Substances Control Act (see also TSCA)
SACB	Science Analysis and Coordination Branch (re: HED of OPP)	TQ	Total quality
SACS	Science Analysis and Coordination Staff (re: EFED of OPP)	TQM	Total quality management
SAES	State agricultural experiment stations	TQSS	Total Quality Specialty Section of the Society of Quality Assurance
SAP	Scientific advisory panel (re: FIFRA)	TRI	Toxics Release Inventory (re: EPCRA)
SARA	Superfund Amendments and Reauthorization Act	TSCA	Toxic Substances Control Act (see also TOSCA)
SB	Systems Branch (re: PMSD of OPP)	TSCATS	TSCA test submissions
SD	Study director	TVOCs	Total volatile organic compounds
SDLC	Software development life cycles	UN	United Nations
SDWA	Safe Drinking Water Act	USC	United States Code
SETAC	Society of Environmental Toxicology & Chemistry	USCDC	United States Centers for Disease Control (see also CDC)
SFIREG	State FIFRA Issues, Research and Evaluation Group	USDA	United States Department of Agriculture
SGML	Standard general markup language (computer language)	USDOE	United States Department of Energy (see also DOE)
SITE	Superfund Innovative Technology Evaluation Program	USDI	United States Department of Interior (see also DI)
SMART	Submission management and review tracking	USEPA	United States Environmental Protection Agency (see also EPA)
SMARTS	Simple Maintenance of ARTS (see also ARTS)	USFDA	United States Food and Drug Administration (see also FDA)
SOP	Standard operating procedure	USFWS	United States Fish and Wildlife Service (see also FWS)
SOT	Society of Toxicology	VAI	Voluntary action indicated
SPI	Standard practice instructions	WEXWPS	Worker Exposure Studies—Worker Protection Standards (re: EPA)
SQA	Society of Quality Assurance	WHO	World Health Organization
SR	Special review (re: OPP; formerly RPAR)	WP	Wettable powders
SRB	Special Review Branch (re: SRRD of OPP)	WSS	Weed Science Society
SRRD	Special Review and Reregistration Division (re: OPP)		
STARS	Submission Tracking And Reporting System	<i>Source:</i>	Courtesy of Society of Quality Assurance, Charlottesville, VA.
STP	Society of Toxicologic Pathologists		

Appendix H: Academic Programs in Toxicology

Many institutions offer academic programs in toxicology at bachelor, master, and doctoral/postdoctoral levels. The Society of Toxicology (SOT) provides a list of these

programs on the SOT website with links to the colleges and universities involved. This list is available to the general public at www.toxicology.org/ai/apt/careerprograms.asp.

Appendix I: Physical/Chemical Information (Tables I.1 through I.6)

Table I.3 gives selected properties of 20- α -amino acids commonly found in proteins. The compounds are listed in alphabetical order by the three-letter symbols. Dissociation constants refer to aqueous solutions at 25°C. M_r = molecular weight; T_m = melting point; pK_a = negative of the logarithm of the dissociation constant for the α -COOH group; pK_b = negative of the logarithm of the dissociation constant for the α -NH₃⁺ group; pK_x = negative of the logarithm of the

dissociation constant for any other group present in the molecule; pI = pH at the isoelectronic point; S = solubility in water at 25°C in units of grams per kilogram of water.

Table I.4 gives approximate pH values for a number of substances of biological importance. All values are rounded off to the nearest tenth and are based on measurements made at 25°C.

TABLE I.1
Decay Constants and Modes for Selected Radionuclides

Nuclide	Half-Life	Decay Mode ^a
Carbon-11	20.3 min	β^+
Carbon-14	5730 years	β^-
Cesium-137	30.0 years	β^-
Chromium-51	27.8 day	EC
Cobalt-57	270 days	EC
Cobalt-60	5.26 years	β^-
Gallium-67	78 h	EC
Gold-198	2.7 days	β^-
Hydrogen-3 (tritium)	12.4 years	β^-
Indium-111	2.8 days	EC
Indium-113m	100 min	IT
Iodine-123	13.3 h	EC
Iodine-125	60 days	EC
Iodine-131	8.05 days	β^-
Iron-59	45 days	β^-
Mercury-197	65 h	EC
Nitrogen-13	10.0 min	β^+
Phosphorus-32	14.3 days	β^-
Oxygen-15	124 s	β^+
Selenium-75	120 days	EC
Sulfur-35	87.4 days	β^-
Technetium-99m	6.0 h	IT
Thallium-201	73 h	EC
Xenon-133	5.3 days	β^-
Ytterbium-169	31.8 days	EC

^a Decay modes include β^- = beta decay; β^+ = positron emission; EC = electron capture; IT = isomeric transition.

TABLE I.2
pK_a Values of Common Drugs

Drug	pK _a	Drug	pK _a
Acetanilide	0.3	Methamphetamine	9.9
Acetazolamide	7.2	Metoprolol	9.6
N-Acetylaminopyrine	0.5	Morphine	8.2
α -Acetylmeadol	8.3	Nortriptyline	9.7
Acetylsalicylic acid	3.5	Orphenadrine	8.4
Aminopyrine	5.0	Oxyphenbutazone	4.7
Amitriptyline	9.4	Papaverine	6.4
Amphetamine	9.9	Pentobarbital	8.1
Amylobarbitol	7.9	Pentazocine	8.8
Aniline	4.6	Phencyclidine	8.5
Antipyrine	1.4	Phenobarbital	7.2
Ascorbic acid	4.1, 11.8	Phenylbutazone	4.5
Atropine	10.0	Phenylpropanolamine	9.1
Barbital	7.5	Physostigmine	8.5
Barbituric acid	4.0	Pilocarpine	6.9
Caffeine	0.8	Probenecid	3.4
Chlorpheniramine	9.0	Propoxyphene	6.3
Chlorpromazine	8.2	Propranolol	9.5
Cocaine	8.5	Pseudoephedrine	9.7
Cycloserine	4.5, 7.4	Quinine	4.1, 8.5
Desipramine	10.2	Reserpine	6.1
Dextromethorphan	8.3	Salicylic acid	3.0
Dicumarol	5.7	Secobarbital	7.9
Diphenhydramine	8.3	Strychnine	8.3
Doxepin	8.0	Sulfadiazine	6.5
Ephedrine	9.9	Sulfaguanidine	>10.00
Ethyl biscoumacetate	3.1	Sulfanilamide	10.4
Glutethimide	11.2	Sulfapyridine	8.4
Hexachlorophene	5.4, 10.9	Sulfathiazole	7.1
Hydrocodone	8.9	Sulfinpyrazone	2.8
Hydromorphone	8.2	Theophylline	0.7
Imipramine	9.5	Thiopental	7.6
Levorphanol	9.2	Tolazoline	10.3
Lidocaine	7.9	Triamterene	6.2
Loxapine	6.6	Trimethobenzamide	8.3
Mecamylamine	11.2	Trimethoprim	7.2
Meperidine	8.7	Vinblastine	5.4, 7.4
Methadone	8.3	Vincristine	5.0, 7.4

TABLE I.3
Properties of Common Amino Acids

Symbol	Name	Molecular Formula	M_r	t_m (°C)	pK_a	pK_b	pK_x	pI	S (g/kg)
Ala	Alanine	$C_3H_7NO_2$	89.09	297	2.33	9.71		6.00	165.0
Arg	Arginine	$C_6H_{14}N_4O_2$	174.20	244	2.03	9.00	12.10	10.76	182.6
Asn	Asparagine	$C_4H_8N_2O_3$	132.12	235	2.16	8.73		5.41	25.1
Asp	Aspartic acid	$C_4H_7NO_4$	133.10	270	1.95	9.66	3.71	2.77	4.95
Cys	Cysteine	$C_3H_7NO_2S$	121.16	240	1.91	10.28	8.14	5.07	v.s.
Glu	Glutamic acid	$C_5H_9NO_4$	147.13	160	2.16	9.58	4.15	3.22	8.61
Gln	Glutamine	$C_5H_{10}N_2O_3$	146.15	185	2.18	9.00		5.65	42
Gly	Glycine	$C_2H_5NO_2$	75.07	290	2.34	9.58		5.97	250.9
His	Histidine	$C_6H_9N_3O_2$	155.16	287	1.70	9.09	6.04	7.59	43.5
Ile	Isoleucine	$C_6H_{13}NO_2$	131.17	284	2.26	9.60		6.02	34.2
Leu	Leucine	$C_6H_{13}NO_2$	131.17	293	2.32	9.58		5.98	22.0
Lys	Lysine	$C_6H_{14}N_2O_2$	146.19	224	2.15	9.16	10.67	9.74	5.8
Met	Methionine	$C_5H_{11}NO_2S$	149.21	281	2.16	9.08		5.74	56
Phe	Phenylalanine	$C_9H_9NO_2$	165.19	283	2.18	9.09		5.48	27.9
Pro	Proline	$C_5H_9NO_2$	115.13	221	1.95	10.47		6.30	1623
Ser	Serine	$C_3H_7NO_3$	105.09	228	2.13	9.05		5.68	421.7
Thr	Threonine	$C_4H_9NO_3$	119.12	256	2.20	8.96		5.60	98.1
Trp	Tryptophan	$C_{11}H_{12}N_2O_2$	204.23	289	2.38	9.34		5.89	13.2
Try	Tyrosine	$C_9H_9NO_3$	181.19	343	2.24	9.04	10.10	5.66	0.46
Val	Valine	$C_5H_{11}NO_2$	117.15	315	2.27	9.52		5.96	88.5

Notes: M_r = molecular weight; t_m = melting point; pK_a , pK_b , pK_c , pK_d = negative of the logarithm of the acid dissociation constants for the COOH and NH_2 groups (and, in some cases, other groups) in the molecule (at 25°C); pI = pH at the isoelectric point; S = solubility in water at 25°C in units of grams of compound per kilogram of water; when quantitative data are not available, the notations sl.s. (for slightly soluble) and v.s. (for very soluble) are used.

TABLE I.4
Approximate pH Values of Biological Materials and Foods

Biological Materials

Blood, plasma, human	7.3–7.5	Gastric contents, human	1.0–3.0	Milk, human	6.6–7.6
Spinal fluid, human	7.3–7.5	Duodenal contents, human	4.8–8.2	Bile, human	6.8–7.0
Blood, whole, dog	6.9–7.2	Feces, human	4.6–8.4		
Saliva, human	6.5–7.5	Urine, human	4.8–8.4		

Foods

Apples	2.9–3.3	Gooseberries	2.8–3.0	Potatoes	5.6–6.0
Apricots	3.6–4.0	Grapefruit	3.0–3.3	Pumpkin	4.8–5.2
Asparagus	5.4–5.8	Grapes	3.5–4.5	Raspberries	3.2–3.6
Bananas	4.5–4.7	Hominy (lye)	6.8–8.0	Rhubarb	3.1–3.2
Beans	5.0–6.0	Jams, fruit	3.5–4.0	Salmon	6.1–6.3
Beer	4.0–5.0	Jellies, fruit	2.8–3.4	Sauerkraut	3.4–3.6
Beets	4.9–5.5	Lemons	2.2–2.4	Shrimp	6.8–7.0
Blackberries	3.2–3.6	Limes	1.8–2.0	Soft drinks	2.0–4.0
Bread, white	5.0–6.0	Maple syrup	6.5–7.0	Spinach	5.1–5.7
Butter	6.1–6.4	Milk, cows	6.3–6.6	Squash	5.0–5.4
Cabbage	5.2–5.4	Olives	3.6–3.8	Strawberries	3.0–3.5
Carrots	4.9–5.3	Oranges	3.0–4.0	Sweet potatoes	5.3–5.6
Cheese	4.8–6.4	Oysters	6.1–6.6	Tomatoes	4.0–4.4
Cherries	3.2–4.0	Peaches	3.4–3.6	Tuna	5.9–6.1
Cider	2.9–3.3	Pears	3.6–4.0	Turnips	5.2–5.6
Corn	6.0–6.5	Peas	5.8–6.4	Vinegar	2.4–3.4
Crackers	6.5–8.5	Pickles, dill	3.2–3.6	Water, drinking	6.5–8.0
Dates	6.2–6.4	Pickles, sour	3.0–3.4	Wine	2.8–3.8
Eggs, fresh white	7.6–8.0	Pimento	4.6–5.2		
Flour, wheat	5.5–6.5	Plums	2.8–3.0		

Source: Lide, D.R., Ed., *Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL, 1990. With permission.

TABLE I.5
Chemical Functional Groups

Acetamido (acetylamino)	$\text{CH}_3\text{CONH}-$
Acetimido (acetylimino)	$\text{CH}_3\text{C(=NH)}-$
Acetoacetamido	$\text{CH}_3\text{COCH}_2\text{CONH}-$
Acetoacetyl	$\text{CH}_3\text{COCH}_2\text{CO}-$
Acetonyl	$\text{CH}_3\text{COCH}_2-$
Acetonylidene	$\text{CH}_3\text{COCH=}$
Acetyl	$\text{CH}_3\text{CO}-$
Acrylyl	$\text{CH}_2=\text{CHCO}-$
Adipyl (from adipic acid)	$-\text{OC(CH}_2)_4\text{CO}-$
Alanyl (from alanine)	$\text{CH}_3\text{CH(NH}_2\text{)CO}-$
B-alanyl	$\text{HN(CH}_2)_2\text{CO}-$
Allophanoyl	$\text{H}_2\text{NCONHCO}-$
Allyl (2-propenyl)	$\text{CH}_2=\text{CHCH}_2-$
Allylidene (2-propenylidene)	$\text{CH}_2=\text{CHCH=}$
Amidino (aminoiminomethyl)	$\text{H}_2\text{NC(=NH)}-$
Amino	$\text{H}_2\text{N}-$
Amyl (pentyl)	$\text{CH}_3(\text{CH}_2)_4-$
Anilino (phenylamino)	$\text{C}_6\text{H}_5\text{NH}-$
Anisidino	$\text{CH}_3\text{OC}_6\text{H}_4\text{NH}-$
Anisyl (from anisic acid)	$\text{CH}_3\text{OC}_6\text{H}_4\text{CO}-$
Anthranoyl (2-aminobenzoyl)	$2-\text{H}_2\text{NC}_6\text{H}_4\text{CO}-$
Arsino	AsH_2-
Azelaoyl (from azelaic acid)	$-\text{OC(CH}_2)_7\text{CO}-$
Azido	N_3-
Azino	$=\text{NN=}$
Azo	$-\text{N=N}-$
Azoxy	$-\text{N(O)N}-$
Benzal	$\text{C}_6\text{H}_5\text{CH=}$
Benzamido (benzylamino)	$\text{C}_6\text{H}_5\text{CONH}-$
Benzhydryl (diphenylmethyl)	$(\text{C}_6\text{H}_5)_2\text{CH}-$
Benzimido (benzylimino)	$\text{C}_6\text{H}_5\text{COO}-$
Benzoxo (benzoyloxy)	$\text{C}_6\text{H}_5\text{COO}-$
Benzoyl	$\text{C}_6\text{H}_5\text{CO}-$
Benzyl	$\text{C}_6\text{H}_5\text{CH}_2-$
Benzylidene	$\text{C}_6\text{H}_5\text{CH=}$
Benzylidyne	$\text{C}_6\text{H}_5\text{C}\equiv$
Biphenyl	$\text{C}_6\text{H}_5\text{C}_6\text{H}_5-$
Biphenylene	$-\text{C}_6\text{H}_4\text{C}_6\text{H}_4-$
Butoxy	$\text{C}_4\text{H}_9\text{O}-$
Sec-butoxy	$\text{C}_2\text{H}_5\text{CH(CH}_3\text{)O}-$
Tert-butoxy	$(\text{CH}_3)_3\text{CO}-$
Butyl	$\text{CH}_3(\text{CH}_2)_3-$
Isobutyl (3-methylpropyl)	$(\text{CH}_3)_2(\text{CH}_2)_2-$
Sec-butyl (1-methylpropyl)	$\text{C}_2\text{H}_5\text{CH(CH}_3\text{)}-$
Tert-butyl (1,1, dimethylethyl)	$(\text{CH}_3)_3\text{C}-$
Butyryl	$\text{C}_3\text{H}_7\text{CO}-$
Caproyl (from caproic acid)	$\text{CH}_3(\text{CH}_2)_4\text{CO}-$
Capryl (from capric acid)	$\text{CH}_3(\text{CH}_2)_6\text{CO}-$
Caprylyl (from caprylic acid)	$\text{CH}_3(\text{CH}_2)_6\text{CO}-$
Carbamido	$\text{H}_2\text{NCONH}-$
Carbamoyl (aminocarbonyl)	$\text{H}_2\text{NCO}-$
Carbamyl (aminocarbonyl)	$\text{H}_2\text{NCO}-$
Carbazoyl (hydrazinocarbonyl)	$\text{H}_2\text{NNHCO}-$
Carbethoxy	$\text{C}_2\text{H}_5\text{O}_2\text{C}-$

TABLE I.5 (continued)
Chemical Functional Groups

Carbobenzoxy	$\text{C}_6\text{H}_5\text{CH}_2\text{O}_2\text{C}-$
Carbonyl	$-\text{C=O}-$
Carboxy	$\text{HOOC}-$
Cetyl	$\text{CH}_3(\text{CH}_2)_{15}-$
Chloroformyl (chlorocarbonyl)	$\text{ClCO}-$
Cinnamyl (3-phenyl-2-propenyl)	$\text{C}_6\text{H}_5\text{CH=CHCH}_2-$
Cinnamoyl	$\text{C}_6\text{H}_5\text{CH=CHCO}-$
Cinnamylidene	$\text{C}_6\text{H}_5\text{CH=CHCH=}$
Cresyl (hydroxymethylphenyl)	$\text{HO(CH}_3\text{)C}_6\text{H}_4-$
Crotoxyl	$\text{CH}_3\text{CH=CHCO}-$
Crotyl (2-butenyl)	$\text{CH}_3\text{CH=CHCH}_2-$
Cyanamido (cyanoamino)	$\text{NCNH}-$
Cyanato	$\text{NCO}-$
Cyano	$\text{NC}-$
Decanedioyl	$-\text{OC(CH}_2)_8\text{CO}-$
Decanoyl	$\text{CH}_3(\text{CH}_2)_8\text{CO}-$
Diazo	$\text{N}_2=$
Diazoamino	$-\text{NHN=N}-$
Disilanyl	$\text{H}_2\text{SiSiH}_2-$
Disiloxanoxo	$\text{H}_3\text{SiOSiH}_2\text{O}-$
Disulfinyl	$-\text{S(O)S(O)}-$
Dithio	$-\text{SS}-$
Enanthyl	$\text{CH}_3(\text{CH}_2)_5\text{CO}-$
Epoxy	$-\text{O}-$
Ethenyl (vinyl)	$\text{CH}_2=\text{CH}-$
Ethynyl	$\text{HC}\equiv\text{C}-$
Ethoxy	$\text{C}_2\text{H}_5\text{O}-$
Ethyl	CH_3CH_2-
Ethylthio	$\text{C}_2\text{H}_5\text{S}-$
Formamido (formylamino)	$\text{HCONH}-$
Formyl	$\text{HCO}-$
Fumaroyl (from fumaric acid)	$-\text{OCCH=CHCO}-$
Furfuryl (2-furanylmethyl)	$\text{OC}_4\text{H}_3\text{CH}_2-$
Furfurylidene (2-furanylmethylene)	$\text{OC}_4\text{H}_3\text{CH=}$
Furyl (furanyl)	OC_4H_3-
Glutamyl (from glutamic acid)	$-\text{OC(CH}_2)_2\text{CH(NH}_2\text{)CO}-$
Glutaryl (from glutaric acid)	$-\text{OC(CH}_2)_3\text{CO}-$
Glycidyl (oxiranylmethyl)	$\text{CH}_2-\text{CHCH}_2-$
Glycinamido	$\text{H}_2\text{NCH}_2\text{CONH}-$
Glycolyl (hydroxyacetyl)	$\text{HOCH}_2\text{CO}-$
Glycyl (aminoacetyl)	$\text{H}_2\text{NCH}_2\text{CO}-$
Glyoxylyl (oxoacetyl)	$\text{HCOCO}-$
Guanidino	$\text{H}_2\text{NC(=NH)NH}-$
Guanyl	$\text{H}_2\text{NC(=NH)}-$
Heptadecanoyl	$\text{CH}_3(\text{CH}_2)_{15}\text{CO}-$
Heptanamido	$\text{CH}_3(\text{CH}_2)_{15}\text{CONH}-$
Heptanedioyl	$-\text{OC(CH}_2)_5\text{CO}-$
Heptanoyl	$\text{CH}_3(\text{CH}_2)_5\text{CO}-$
Hexadecanoyl	$\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$
Hexamethylene	$-(\text{CH}_2)_6-$
Hexanedioyl	$-\text{OC(CH}_2)_4\text{CO}-$
Hippuryl (N-benzoylglycyl)	$\text{C}_6\text{H}_5\text{CONHCH}_2\text{CO}-$
Hydantoyl	$\text{H}_2\text{NCONHCH}_2\text{CO}-$
Hydrazino	$\text{N}_2\text{NNH}-$

TABLE I.5 (continued)
Chemical Functional Groups

Hydrazo	–HNNH–
Hydrocinnamoyl	$C_6H_5(CH_2)_2CO-$
Hydroperoxy	HOO–
Hydroxyamino	HONH–
Hydroxy	HO–
Imino	HN=
Iodoso	OI–
Isoamyl (isopentyl)	$(CH_3)_2CH(CH_2)_2-$
Isobutenyl (2-methyl-1-propenyl)	$(CH_3)_2C=CH-$
Isobutoxy	$(CH_3)_2CHCH_2O-$
Isobutyl	$(CH_3)_2CHCH_2-$
Isobutylidene	$(CH_3)_2CHCH=$
Isobutyryl	$(CH_3)_2CHCO-$
Isocyanato	OCN–
Isocyano	CN–
Isohexyl	$(CH_3)_2CH(CH_2)_3-$
Isoleucyl (from isoleucine)	$C_7H_{13}CH(CH_3)CH(NH_2)CO-$
Isonitroso	HON=
Isopentyl	$(CH_3)_2CH(CH_2)_2-$
Isopentylidene	$(CH_3)_2CHCH_2CH=$
Isopropenyl	$H_2C=C(CH_3)-$
Isopropoxy	$(CH_3)_2CHO-$
Isopropyl	$(CH_3)_2CH-$
Isopropylidene	$(CH_3)_2C=$
Isothiocyanato (isothiocyano)	SCN–
Isovaleryl (from isovaleric acid)	$(CH_3)_2CHCH_2CO-$
Keto (oxo)	O=
Lactyl (from lactic acid)	$CH_3CH(OH)CO-$
Lauroyl (from lauric acid)	$CH_3(CH_2)_{10}CO-$
Leucyl (from leucine)	$(CH_3)_2CHCH_2CH(NH_2)CO-$
Levulinyl (From levulinic acid)	$CH_3CO(CH_2)_2CO-$
Malonyl (from malonic acid)	$-OCCH_2CO-$
Mandetyl (from mandelic acid)	$C_6H_5CH(OH)CO-$
Mercapto	HS–
Methacrylyl (from methacrylic acid)	$CH_2=C(CH_3)CO-$
Methallyl	$CH_2=C(CH_3)CH_2-$
Methionyl (from methionine)	$CH_3SCH_2CH_2CH(NH_2)CO_2-$
Methoxy	CH_3O-
Methyl	H_3C-
Methylene	$H_2C=$
Methylenedioxy	$-OCH_2O-$
Methylenedisulfonyl	$-O_2SCH_2SO_2-$
Methylol	$HOCH_2-$
Methylthio	CH_2S-
Myristyl (from myristic acid)	$CH_3(CH_2)_{12}CO-$
Naphthol	$(C_{10}H_7)CH=$
Naphthobenzyl	$(C_{10}H_7)CH_2-$
Naphthoxy	$(C_{10}H_7)O-$
Naphthyl	$(C_{10}H_7)-$
Naphthylidene	$(C_{10}H_6)=$
Neopentyl	$(CH_3)_3CCH_2-$
Nitramino	O_2NNH-
Nitro	O_2N-
Nitrosamino	$ONNH-$

TABLE I.5 (continued)
Chemical Functional Groups

Nitrosimino	ONN=
Nitroso	ON–
Nonanoyl (from nonanoic acid)	$CH_3(CH_2)_7CO-$
Oleyl (from oleic acid)	$CH_3(CH_2)_7CH=CH(CH_2)_7CO-$
Oxalyl (from oxalic acid)	–OCCO–
Oxamido	$H_2NCOCONH-$
Oxo (keto)	O=
Palmityl (from palmitic acid)	$CH_3(CH_2)_{14}CO-$
Pelargonyl (from pelargonic acid)	$CH_3(CH_2)_7CO-$
Pentamethylene	– $(CH_2)_5-$
Pentyl	$CH_3(CH_2)_4-$
Phenacyl	$C_6H_5COCH_2-$
Phenacylidene	$C_6H_5COCH=$
Phenanthryl	$(C_{14}H_9)-$
Phenethyl	$C_6H_5CH_2CH_2-$
Phenoxy	C_6H_5O-
Phenyl	C_6H_5-
Phenylene	– C_6H_4-
Phenylenedioxy	– OC_6H_4O-
Phosphino	H_2P-
Phosphinyl	$H_2P(O)-$
Phospho	O_2P-
Phosphono	$(HO)_2P(O)-$
Phthalyl (from phthalic acid)	$1,2-C_6H_4(CO-)_2$
Picryl (2,4,6-trinitrophenyl)	$2,4,6-(NO_2)_2C_6H_2-$
Pimelyl (from pimelic acid)	– $OC(CH_2)_5CO-$
Piperidino	$C_5H_{10}N-$
Piperidyl (piperidinyl)	$(C_5H_{10}N)-$
Piperonyl	$3,4-(CH_2O)_2C_6H_3CH_2-$
Pivalyl (from pivalic acid)	$(CH_3)_3CCO-$
Prenyl (3-methyl-2-butenyl)	$(CH_3)_2C=CHCH_2-$
Propargyl (2-propynyl)	$HC\equiv CCH_2-$
Propenyl	$CH_2=CHCH_2-$
iso-Propenyl	$(CH_3)_2C=$
Propionyl	CH_3CH_2CO-
Propoxy	$CH_3CH_2CH_2O-$
Propyl	$CH_3CH_2CH_2-$
iso-Propyl	$(CH_3)_2CH-$
Propylidene	$CH_3CH_2CH=$
Pyridino	C_5H_5N-
Pyridyl (pyridinyl)	$(C_5H_4N)-$
Pyrryl (pyrrolyl)	$(C_3H_4N)-$
Salicyl (2-hydroxybenzoyl)	$2-HOC_6H_4CO-$
Selenyl	HSe–
Seryl (from serine)	$HOCH_2CH(NH_2)CO-$
Siloxy	H_3SiO-
Silyl	H_3Si-
Silylene	$H_2Si=$
Sorbyl (from sorbic acid)	$CH_3CH=CHCH=CHCO-$
Stearyl (from stearic acid)	$CH_3(CH_2)_{16}CO-$
Styryl	$C_6H_5CH=CH-$
Suberyl (from suberic acid)	– $OC(CH_2)_6CO-$
Succinamyl	$H_2NCOCH_2CH_2CO-$
Succinyl (from succinic acid)	– $OCCH_2CH_2CO-$

(continued)

TABLE I.5 (continued)
Chemical Functional Groups

Sulfamino	HOSO ₂ NH–
Sulfamyl	H ₂ NSO–
Sulfanilyl	4-H ₂ NC ₆ H ₄ SO ₂ –
Sulfeno	HOS–
Sulphydryl (mercapto)	HS–
Sulfinyl	OS=
Sulfo	HO ₃ S–
Sulfonyl	–SO ₂ –
Terephthalyl	1,4–C ₆ H ₄ (CO–) ₂
Tetramethylene	–(CH ₂) ₄ –
Thenyl	(C ₄ H ₃ S)CH–
Thienyl	(C ₄ H ₃ S)–
Thiobenzoyl	C ₆ H ₅ CS–
Thiocarbamyl	H ₂ NCS–
Thiocarbonyl	–CS–
Thiocarboxy	HOSC–
Thiocyanato	NCS–
Thionyl (sulfinyl)	–SO–
Thiophenacyl	C ₆ H ₅ CSCH ₂ –
Thiurain(aminothioxomethyl)	H ₂ NCS–
Threonyl (from threonine)	CH ₃ CH(OH)CH(NH ₂)CO–
Toluidino	CH ₃ C ₆ H ₄ NH–
Toluyyl	CH ₃ C ₆ H ₄ CO–
Tolyl (methylphenyl)	CH ₃ C ₆ H ₄ –
α-tolyl	C ₆ H ₅ CH ₂ –
Tolylene (methylphenylene)	(CH ₃ C ₆ H ₃)=
α-tolylene	C ₆ H ₅ CH=
Tosyl [(4-methylphenyl) sulfonyl]	4–CH ₃ C ₆ H ₄ SO ₂ –
Triazano	H ₂ NNHNH–
Trimethylene	–(CH ₂) ₃ –
Triphenylmethyl (trityl)	(C ₆ H ₅) ₃ C–
Tyrosyl (from tyrosine)	4–HOC ₆ H ₄ CH ₂ CH(NH ₂)CO–
Ureido	H ₂ NCONH–
Valeryl (from valeric acid)	C ₄ H ₉ CO
Valyl (from valine)	(CH ₃) ₂ CHCH(NH ₂)CO–
Vinyl	CH ₂ =CH–
Vinylidene	CH ₂ =C=
Xenyl (biphenyl)	C ₆ H ₅ C ₆ H ₄ –
Xylidino	(CH ₃) ₂ C ₆ H ₃ NH–
Xylyl (dimethylphenyl)	(CH ₃) ₂ C ₆ H ₃ –
Xylylene	–CH ₂ C ₆ H ₄ CH ₂ –

Source: From Lide, D.R., Ed., *CRC Handbook of Chemistry and Physics*, 73rd edn., CRC Press, Boca Raton, FL, 1992–1993. With permission.

TABLE I.6
Standard Atomic Weights (1987)

Name	Symbol	No.	Atomic Weight	Footnotes		
Actinium ^a	Ac	89				b
Aluminum	Al	13	26.981539(5)			
Americium ^a	Am	95				b
Antimony (Stibium)	Sb	51	121.75(3)			
Argon	Ar	18	39.948(1)	c		d
Arsenic	As	33	74.92159(2)			
Astatine ^a	At	85				b
Barium	Ba	56	137.327(7)			
Berkelium ^a	Bk	97				b
Beryllium	Be	4	9.012182(3)			
Bismuth	Bi	83	208.98037(3)			
Boron	B	5	10.811(5)	c	e	d
Bromine	Br	35	79.904(1)			
Cadmium	Cd	48	112.411(8)	c		
Cesium	Cs	55	132.90543(5)			
Calcium	Ca	20	40.078(4)	c		
Californium ^a	Cf	98				b
Carbon	C	6	12.011(1)			d
Cerium	Ce	58	140.115 (4)	c		
Chlorine	Cl	17	35.4527(9)			
Chromium	Cr	24	51.9961(6)			
Cobalt	Co	27	58.93320(1)			
Copper	Cu	29	63.546(3)			d
Curium ^a	Cm	96				b
Dysprosium	Dy	66	162.50(3)	c		
Einsteinium ^a	Es	99				b
Erbium	Er	68	167.26(3)	c		
Europium	Eu	63	151.965(9)	c		
Fermium ^a	Fm	100				b
Fluorine	F	9	18.9984032(9)			
Francium ^a	Fr	87				b
Gadolinium	Gd	64	157.25(3)	c		
Gallium	Ga	31	69.723(1)			
Germanium	Ge	32	72.61(2)			
Gold	Au	79	196.96654(3)			
Hafnium	Hf	72	178.49(2)			
Helium	He	2	4.002602(2)	c		d
Holmium	Ho	67	164.93032(3)			
Hydrogen	H	1	1.00794(7)	c	e	d
Indium	In	49	114.82(1)			
Iodine	I	53	126.90447(3)			
Iridium	Ir	77	192.22(3)			
Iron	Fe	26	55.847(3)			
Krypton	Kr	36	83.80(1)	c	e	
Lanthanum	La	57	138.9055(2)	c		
Lawrencium ^a	Lr	103				b
Lead	Pb	82	207.2(1)	c		d
Lithium	Li	3	6.941(2)	c	e	d
Lutetium	Lu	71	174.967(1)	c		

TABLE I.6 (continued)
Standard Atomic Weights (1987)

Name	Symbol	No.	Atomic Weight	Footnotes
Magnesium	Mg	12	24.3050(6)	
Manganese	Mn	25	54.93805(1)	
Mendelevium ^a	Md	101		b
Mercury	Hg	80	200.59(3)	
Molybdenum	Mo	42	95.94(1)	
Neodymium	Nd	60	144.24(3)	c
Neon	Ne	10	20.1797(6)	c e
Neptunium ^a	Np	93		b
Nickel	Ni	28	58.69(1)	
Niobium	Nb	41	92.90638(2)	
Nitrogen	N	7	14.00674(7)	c d
Nobelium ^a	No	102		b
Osmium	Os	76	190.2(1)	c
Oxygen	O	8	15.9994(3)	c d
Palladium	Pd	46	105.42(1)	c
Phosphorus	P	15	30.973762(4)	
Platinum	Pt	78	195.08(3)	
Plutonium ^a	Pu	94		b
Polonium ^a	Po	84		b
Potassium (Kalium)	K	19	39.0983(1)	
Praseodymium	Pr	59	140.90765(3)	
Promethium ^a	Pm	61		b
Protactinium ^a	Pa	91		b
Radium ^a	Ra	88		b
Radon ^a	Rn	86		b
Rhenium	Re	75	186.207(1)	
Rhodium	Rh	45	102.90550(3)	
Rubidium	Rb	37	85.4678(3)	c
Ruthenium	Ru	44	101.07(2)	c
Samarium	Sm	62	150.36(3)	c
Scandium	Sc	21	44.955910(9)	
Selenium	Se	34	78.96(3)	
Silicon	Si	14	28.0855(3)	d
Silver	Ag	47	107.8682(2)	c
Sodium (Natrium)	Na	11	22.989768(6)	
Strontium	Sr	38	87.62(1)	c d
Sulfur	S	16	32.066(6)	d
Tantalum	Ta	73	180.9479(1)	
Technetium ^a	Tc	43		b
Tellurium	Te	52	127.60(3)	c
Terbium	Tb	65	158.92534(3)	
Thallium	Tl	81	204.3833(2)	
Thorium ^a	Th	90	232.0381(1)	c f
Thulium	Tm	69	168.93421(3)	
Tin	Sn	50	118.710(7)	c
Titanium	Ti	22	47.88(3)	
Tungsten (Wolfram)	W	74	183.85(3)	
Unnilquadium	Unq	104		b
Unnilpentium	Unp	105		b

TABLE I.6 (continued)
Standard Atomic Weights (1987)

Name	Symbol	No.	Atomic Weight	Footnotes
Unnilhexium	Unh	106		b
Unnilseptium	Uns	107		b
Uranium ^a	U	92	238.0289(1)	c e f
Vanadium	V	23	50.9415(1)	
Xenon	Xe	54	131.29(2)	c e
Ytterbium	Yb	70	173.04(3)	c
Yttrium	Y	39	88.90585(2)	
Zinc	Zn	30	65.39(2)	
Zirconium	Zr	40	91.224(2)	c

Source: Lide, D.R., Ed., *CRC Handbook of Chemistry and Physics*, 73rd edn., CRC Press, Boca Raton, FL, 1992.

Notes: Scaled to Ar (12C) = 12. The atomic weights of many elements are not invariant but depend on the origin and treatment of the material. The types of variation to be expected for individual elements are elaborated here. The values of atomic weight given here apply to elements as they exist naturally on earth.

^a Element has no stable nuclides.

^b Radioactive element that lacks a characteristic terrestrial isotopic composition.

^c Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the table may exceed the implied uncertainty.

^d Range in isotopic composition of normal terrestrial material prevents a more precise atomic weight being given: the tabulated atomic weight value should be applicable to any normal material.

^e Modified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic separation. Substantial deviations in atomic weight of the element from that given in the table can occur.

^f An element, without stable nuclide(s), exhibiting a range of characteristic terrestrial compositions of long-lived radionuclide(s) such that a meaningful atomic weight can be given.

REFERENCES

- Fasman, G. D., Ed., *Practical Handbook of Biochemistry and Molecular Biology*, CRC Press, Boca Raton, FL, 1989.
- Hinz, H. J., Ed., *Thermodynamic Data for Biochemistry and Biotechnology*, Springer-Verlag, Heidelberg, Germany, 1986.
- Lide, D. R., Ed., *Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL, 1990.
- Lide, D. R., Ed., *CRC Handbook of Chemistry and Physics*, 73rd edn., CRC Press, Boca Raton, FL, 1992.
- Smith, E. L. et al., *Principles of Biochemistry*, 7th edn., McGraw Hill, New York, 1983.

Glossary: By Subject

CARCINOGENESIS*

Adduct: The covalent linkage or addition product between an alkylating agent and cellular macromolecules such as protein, RNA, and DNA.

Alkylating agent: A chemical compound that has positively charged (electron-deficient) groups that can form covalent linkages with negatively charged portions of biological molecules such as DNA. The covalent linkage is referred to as an adduct and may have mutagenic or carcinogenic effects on the organism. The alkyl species is the radical that results when an aliphatic hydrocarbon loses one hydrogen atom to become electron deficient. Alkylating agents react primarily with guanine, adding their alkyl group to N7 of the purine ring.

Altered focus: A histologically identifiable clone of cells within an organ that differs phenotypically from the normal parenchyma. Foci of altered cells usually result from increased cellular proliferation, represent clonal expansions of initiated cells, and are frequently observed in multistage animal models of carcinogenesis. Foci of cellular alteration are most commonly observed in the liver of carcinogen-treated rodents and are believed by some to represent preneoplastic lesions.

Benign: A classification of anticipated biological behavior of neoplasms in which the prognosis for survival is good. Benign neoplasms grow slowly, remain localized, and usually cause little harm to the patient.

Choristoma: A mass of well-differentiated cells from one organ included within another organ, for example, adrenal tissue present in the lung.

Chromosomal aberration: A numerical or structural chromosomal abnormality.

Cocarcinogen: An agent not carcinogenic alone but that potentiates the effect of a known carcinogen.

Cocarcinogenesis: The augmentation of neoplasm formation by simultaneous administration of a genotoxic carcinogen and an additional agent (cocarcinogen) that has no inherent carcinogenic activity by itself.

Direct carcinogen: Carcinogens that have the necessary structure to directly interact with cellular constituents and cause neoplasia. Direct acting carcinogens do not require metabolic conversion by the host to be active. They are considered genotoxic because they typically undergo covalent binding to DNA.

Dysplasia: Disordered tissue formation characterized by changes in size, shape, and orientational relationships of adult types of cells. Primarily seen in epithelial cells.

Epigenetic: Change in phenotype without a change in DNA structure. One of two main mechanisms of carcinogen action, epigenetic carcinogens are nongenotoxic, that is, they do not form reactive intermediates that interact with genetic material in the process of producing or enhancing neoplasm formation.

Genotoxic carcinogen: An agent that interacts with cellular DNA either directly in its parent form (direct carcinogen) or after metabolic biotransformation.

Hyperplasia: A numerical increase in the number of phenotypically normal cells within a tissue or organ.

Hypertrophy: Increase in the size of an organelle, cell, tissue, or organ within a living organism. To be distinguished from hyperplasia, hypertrophy refers to an increase in size rather than an increase in number. Excessive hyperplasia in a tissue may produce hypertrophy of the organ in which that tissue occurs.

In situ carcinoma: A localized intraepithelial form of epithelial cell malignancy. The cells possess morphological criteria of malignancy but have not yet gone beyond the limiting basement membrane.

Initiation: The first step in carcinogenesis whereby limited exposure to a carcinogenic agent produces a latent but heritable alteration in a cell, permitting its subsequent proliferation and development into a neoplasm after exposure to a promoter.

Initiator: A chemical, physical, or biological agent that is capable of irreversibly altering the genetic component (DNA) of the cell. While initiators are generally considered to be carcinogens, they are typically used at low noncarcinogenic doses in two-stage initiation–promotion animal model systems. Frequently referred to as a “tumor initiator.”

Malignant: A classification of anticipated biological behavior of neoplasms in which the prognosis for survival is poor. Malignant neoplasms grow rapidly, invade, destroy, and are usually fatal.

Metaplasia: The substitution in a given area of one type of fully differentiated cell for the fully differentiated cell type normally present in that area, for example, squamous epithelium replacing ciliated epithelium in the respiratory airways.

Metastasis: The dissemination of cells from a primary neoplasm to a noncontiguous site and their growth therein. Metastases arise by dissemination of cells from the primary neoplasm via the vascular or lymphatic system and are an unequivocal hallmark of malignancy.

* Maronpot (1991). Reprinted in part with permission.

Mitogenesis: The generation of cell division or cell proliferation.

Maximum tolerated dose (MTD): Refers to the maximum amount of an agent that can be administered to an animal in a carcinogenicity test without adversely affecting the animal due to toxicity other than carcinogenicity. Examples of having exceeded the MTD include excessive early mortality, excessive loss of body weight, production of anemia, production of tissue necrosis, and overloading of the metabolic capacity of the organism.

Mutation: A structural alteration of DNA that is hereditary and gives rise to an abnormal phenotype. A mutation is always a change in the DNA base sequence and includes substitutions, additions, rearrangements, or deletions of one or more nucleotide bases.

Oncogene: The activated form of a proto-oncogene. Oncogenes are associated with the development of neoplasia.

Preneoplastic lesion: A lesion usually indicative that the organism has been exposed to a carcinogen. The presence of preneoplastic lesions indicates that there is enhanced probability for the development of neoplasia in the affected organ. Preneoplastic lesions are believed to have a high propensity to progress to neoplasia.

Procarcinogen: An agent that requires bioactivation in order to give rise to a direct acting carcinogen. Without metabolic activation, these agents are not carcinogenic.

Progression: Processes associated with the development of an initiated cell to a biologically malignant neoplasm. Sometimes used in a more limited sense to describe the process whereby a neoplasm develops from a benign to a malignant proliferation or from a low-grade to a high-grade malignancy. Progression is that stage of neoplastic development characterized by demonstrable changes associated with increased growth rate, increased invasiveness, metastases, and alterations in biochemical and morphological characteristics of a neoplasm.

Promoter: *Use in multistage carcinogenesis*—an agent that is not carcinogenic itself but when administered after an initiator of carcinogenesis stimulates the clonal expansion of the initiated cell to produce a neoplasm. *Use in molecular biology*—a DNA sequence that initiates the process of transcription and is located near the beginning of the first exon of a structural gene.

Promotion: The enhancement of neoplasm formation by the administration of a carcinogen followed by an additional agent (promoter) that has no intrinsic carcinogenic activity by itself.

Protooncogene: A normal cellular structural gene that, when activated by mutations, amplifications, rearrangements, or viral transduction, functions as an oncogene and is associated with the development

of neoplasia. Proto-oncogenes regulate functions related to normal growth and differentiation of tissues.

Regulatory gene: A gene that controls the activity of a structural gene or another regulatory gene. Regulatory genes usually do not undergo transcription into messenger RNA.

Sister chromatid exchange: The morphological reflection of an interchange between DNA molecules at homologous loci within a replicating chromosome.

Somatic cell: A normal diploid cell of an organism as opposed to a germ cell, which is haploid. Most neoplasms are believed to begin when a somatic cell is mutated.

Transformation: Typically refers to tissue culture systems where there is conversion of normal cells into cells with altered phenotypes and growth properties. If such cells are shown to produce invasive neoplasms in animals, malignant transformation is considered to have occurred.

Ultimate carcinogen: That form of the carcinogen that actually interacts with cellular constituents to cause the neoplastic transformation. The final product of metabolism of the procarcinogen.

CLINICAL PATHOLOGY

Activated partial thromboplastin time: A measure of the relative activity of factors in the intrinsic clotting sequence and the common pathway necessary in normal blood coagulation.

Alanine aminotransferase (ALT): An enzyme, primarily of liver origin, whose blood levels can rise in response to hepatocellular toxicity. Also known as serum glutamic pyruvic transaminase (SGPT).

Albumin: The most abundant blood protein synthesized by the liver.

Alkaline phosphatase: An enzyme whose blood levels can rise in response to hepatobiliary disease or increased osteoblastic (bone cell) activity. Serum alkaline phosphatase activity can decrease in fasted rats because the intestinal isozyme is an important component of serum enzyme activity.

Anemia: Any conditions in which RBC count, hemoglobin concentration, and hematocrit are reduced.

Anisocytosis: Variations in the size of red blood cells.

Aspartate aminotransferase (AST): An enzyme whose blood levels can rise in response to hepatotoxicity, muscle damage, or hemolysis. Also known as serum glutamic oxaloacetic transaminase (SGOT).

Azotemia: An increase in serum urea nitrogen and/or creatinine levels.

Creatine kinase (CK): An enzyme that is concentrated in skeletal muscle, brain, and heart tissue.

Creatinine: The end product of creatine metabolism in muscle. Elevated blood levels can indicate renal (glomerular) injury.

Fibrinogen: A glycoprotein that is involved in the formation of fibrin.

Gamma glutamyltransferase (γ GT): An enzyme of liver origin, whose blood concentration can be elevated in hepatobiliary disease.

Globulin: A group of blood proteins synthesized by lymphatic tissue in the liver.

Hemolysis: The destruction of red blood cells resulting in the liberation of hemoglobin into plasma.

Icteric: Relating to a jaundiced condition, typically as a result of elevated serum bilirubin levels.

Lactate dehydrogenase: An enzyme found in several organs, including liver, kidney, heart, and skeletal muscle.

Mean corpuscular hemoglobin (MCH): The average amount of hemoglobin per red blood cell.

Mean corpuscular hemoglobin concentration (MCHC): The average hemoglobin concentration per red blood cell.

Mean corpuscular volume (MCV): The average size of the red blood cell.

Methemoglobin: Oxidized hemoglobin incapable of carrying oxygen.

Packed cell volume: The percent of blood that contains RBC components; synonymous with hematocrit.

Poikilocytosis: Variations in the shape of red blood cells.

Polychromasia: Increased basophilic staining of erythrocytes.

Polycythemia: An increase in the number of red blood cells.

Prothrombin time: A measure of the relative activity of factors in the extrinsic clotting sequence and the common pathway necessary in normal blood coagulation.

Reticulocyte: An immature (polychromatic) erythrocyte.

Reticulocytosis: Increased numbers of reticulocytes in the circulation, typically seen in response to regenerative anemia.

Sorbitol dehydrogenase (SDH): An enzyme of liver origin, whose blood concentration rises in response to hepatocellular injury.

Triglycerides: Synthesized primarily in the liver and intestine; the major form of lipid storage.

Urea nitrogen (BUN): The end product of protein catabolism. Blood levels can rise after renal (glomerular) injury.

DERMAL TOXICOLOGY

Acanthosis: Hypertrophy of the stratum spinosum and granulosum.

Blanching: To take color from, to bleach. Characterized by a white or pale discoloration of the exposure area due to decreased blood flow to the skin (ischemia).

Contact dermatitis: A delayed type of induced sensitivity (allergy) of the skin with varying degrees of erythema, edema, and vesiculation, resulting from cutaneous contact with a specific allergen.

Contact urticaria: Wheal-and-flare response elicited with 30–60 min after cutaneous exposure to test substance. May be IgE mediated or nonimmunologically mediated.

Corrosion: Direct chemical action on normal living skin that results in its disintegration and irreversible alteration at the site of contact. Corrosion is manifested by ulceration and necrosis with subsequent scar formation.

Cumulative irritation: Primary irritation resulting from repeated exposures to materials that do not in themselves cause acute primary irritation.

Dermatitis: Inflammation of the skin.

Desquamation: The shedding of the cuticle in scales or the outer layer of any surface. To shred, peel, or scale off, as the casting off of the epidermis in scales or shred or the shedding of the outer layer of any surface.

Eczema: Inflammatory condition in which the skin becomes red and small vesicles, crusts, and scales develop.

Edema: An excessive accumulation of serous fluid or water in cells, tissues, or serous cavities.

Erythema: An inflammatory redness of the skin, as caused by chemical poisoning or sunburn, usually a result of congestion of the capillaries.

Eschar: A dry scab, thick coagulated crust or slough formed on the skin as a result of a thermal burn or by the action of a corrosive or caustic substance.

Exfoliation: To remove in flakes or scales, peel. To cast off in scales, flakes, or the like. To come off or separate, as scales, flakes, sheets, or layers. Detachment and shedding of superficial cells of an epithelium or from any tissue surface. Scaling or desquamation of the horny layer of epidermis, which varies in amount from minute quantities to shedding the entire integument.

Hyperkeratosis: Hypertrophy and thickening of the stratum corneum.

Irritant: A substance that causes inflammation and other evidences of irritation, particularly of the skin, on first contact or exposure; a reaction of irritation not dependent on a mechanism of sensitization.

Irritation: A local reversible inflammatory response of normal living skin to direct injury caused by a single application of a toxic substance, without the involvement of an immunological mechanism.

Necrosis: Pathological death of one or more cells or of a portion of tissue or organ, resulting from irreversible damage.

Nonocclusive: The site of application of test substance is open to the air.

Occlusive: A bandage or dressing that covers the skin and excludes it from air. Prevents loss of a test substance by evaporation and by increasing tissue penetration.

Photoallergy: An increased reactivity of the skin to ultraviolet (UV) and/or visible radiation produced by a chemical agent on an immunological basis. Previous

allergy sensitized by exposure to the chemical agent and appropriate radiation is necessary. The main role of light in photoallergy appears to be in the conversion of the hapten to a complete allergen.

Photoirritation: Irritation resulting from light-induced molecular changes in the structure of chemicals applied to the skin.

Photosensitization: Sensitization of the skin to UV light, usually due to the action of certain drugs, plants, or other substances; may occur shortly after administration of the substance or may occur only after latent period of days to months. The processes whereby foreign substances, absorbed either locally into the skin or systemically, may be subjected to photochemical reactions within the skin, either leading to chemically induced photosensitivity reactions or altering the "normal" pathologic effects of light. UV-A is usually responsible for most photosensitivity reactions.

Semiocclusive: The site of application of test substance is covered; however, movement of air through covering is not restricted.

Sensitization (allergic contact dermatitis): An immunologically mediated cutaneous reaction to a substance.

Superficial sloughing: Characterized by dead tissue separated from a living structure. Any outer layer or covering that is shed. Necrosed tissue separated from the living structure.

Ulceration: The development of an inflammatory, often suppurating lesion, on the skin or an internal mucous surface of the body caused by superficial loss of tissue, resulting in necrosis of the tissue.

ECOTOXICOLOGY*

Bioaccumulation: General term describing a process by which chemicals are taken up by aquatic organisms directly from water as well as from exposure through other routes, such as consumption of food and sediment containing chemicals.

Bioaccumulation factor (BAF): The ratio of tissue chemical residue to chemical concentration in an external environmental phase (i.e., water, sediment, or food). BAF is measured as steady state in situations where organisms are exposed from multiple sources (i.e., water, sediment, and food), unless noted otherwise.

Biochemical oxygen demand (BOD): Sometimes called *biological oxygen demand*, a measure of the rate at which molecular oxygen is consumed by microorganisms during oxidation of organic matter. The standard test is the 5-day BOD test, in which the amount of dissolved oxygen required for oxidation over a 5-day period is measured. The results are measured in mg of oxygen/L (mg/L) or parts per million (ppm).

Bioconcentration: A process by which there is a net accumulation of a chemical directly from water into aquatic organisms resulting from simultaneous uptake (e.g., by gill or epithelial tissue) and elimination.

Bioconcentration factor (BCF): A term describing the degree to which a chemical can be concentrated in the tissues of an organism in the aquatic environment as a result of exposure to waterborne chemical. At steady state during the uptake phase of a bioconcentration test, the BCF is a value that is equal to the concentration of a chemical in one or more tissues of the exposed aquatic organisms divided by the average exposure water concentration of the chemical in the test.

Biodegradation: The transformation of a material resulting from the complex enzymatic action of microorganisms (e.g., bacteria, fungi). It usually leads to disappearance of the parent chemical structure and to the formation of smaller chemical species, some of which are used for cell anabolism. Although typically used with reference to microbial activity, it may also refer to general metabolic breakdown of a substance by any living organism.

Chemical oxygen demand (COD): COD is measured instead of BOD when organic materials are not easily degraded by microorganisms. Strong oxidizing agents (e.g., potassium permanganate) are used to enhance oxidation. COD values will be larger than BOD values.

EC₅₀ (median effective concentration): The concentration of chemical in water to which test organisms are exposed that is estimated to be effective in producing some sublethal response in 50% of the test organisms. The EC₅₀ is usually expressed as a time-dependent value (e.g., 24 h or 96 h EC₅₀). The sublethal response elicited from the test organisms as a result of exposure to the chemical must be clearly defined (e.g., test organisms may be immobilized, lose equilibrium, or undergo physiological or behavioral changes).

Fate: Disposition of a material in various environmental compartments (e.g., soil or sediment, water, air, biota) as a result of transport, transformation, and degradation.

LC₅₀ (median lethal concentration): The concentration of chemical in water to which test organisms are exposed that is estimated to be lethal to 50% of the test organisms. The LC₅₀ is often expressed as a time-dependent value (e.g., 24 h or 96 h LC₅₀).

Maximal acceptable toxicant concentration (MATC): The hypothetical toxic threshold concentration lying in a range bounded at the lower end by the highest tested concentration having no observed effect (NOEC) and at the higher end by the lowest concentration having a statistically significant toxic effect (LOEC) in a life cycle (full chronic) or a partial life cycle (partial chronic) test. This can be represented by NOEC < MATC < LOEC.

* Rand (1995). With permission.

Octanol–water partition coefficient (K_{ow}): The ratio of the solubility of a chemical in n-octanol and water at steady state; also expressed as P . The logarithm of P or K_{ow} (i.e., $\log P$ or K_{ow}) is used as an indication of the propensity of a chemical for bioconcentration by aquatic organisms.

TL_m or TL₅₀ (median tolerance limit): The concentration of material in water at which 50% of the test organisms survive after a specified time of exposure. The TL_m (or TL₅₀) is usually expressed as a time-dependent value (e.g., 24 h or 96 h TL₅₀).

GENETIC TOXICOLOGY

Aneuploidy: An abnormal number of chromosomes in a cell or organism that is not an exact multiple of the haploid number.

Base substitution: The substitution of one or more base(s) for another in the nucleotide sequence.

Clastogen: An agent that produces structural changes of chromosomes.

Frameshift mutation: A mutation in the genetic code in which one base or two adjacent bases are inserted or deleted to the nucleotide sequence of a gene.

Gene mutation: A detectable permanent change (point mutation, insertion, or deletion) within a single gene or its regulating sequences.

Micronucleus: A microscopically detectable particle in a cell that contains nuclear DNA, usually 1/20th to 1/5th the size of the main nucleus. It may be composed of a broken centric or acentric part of a chromosome or a whole chromosome.

Mitotic index: The ratio of the number of cells in a population in various stages of mitosis to the number of cell in the population not in mitosis.

Plasmid: An autonomously replicating DNA molecule distinct from the normal genome. A plasmid may insert into the host chromosome or form an extra chromosomal element.

Point mutation: Change in the genetic code, usually confined to a single base pair.

Unscheduled DNA synthesis (UDS): DNA synthesis that occurs at some stage in the cell cycle other than S-phase in response to DNA damage and is usually associated with DNA excision repair.

IMMUNOTOXICOLOGY

Antibody-dependent cell-mediated cytotoxicity (ADCC): Cell-mediated immunity (CMI) in which a specific antibody binds to a target cell, targeting it for cytolytic activity by an effector cell (generally a macrophage or natural killer [NK] cell).

Adjuvant (immunological): A material that enhances an immune response but does not confer immunity by itself. Examples include oil emulsions, aluminum salts, and toll-like receptor agonists. Complete

Freund's Adjuvant (CFA) was first developed by Jules Freund in the 1940s and contains a water-in-oil emulsion and mycobacterial cell fragments. Incomplete Freund's Adjuvant does not contain the mycobacterial cell fragments.

Allogeneic: From a different genetic background. In the context of immunotoxicology, this usually refers to the use of genetically dissimilar cells in in vitro assays to elicit a cell-mediated immune reaction.

Antibody complex: Macromolecules produced by plasma cells that recognize specific antigens.

Antibodies: Are also referred to as immunoglobulins (Ig). They consist of two basic units: the antigen-binding fragment (Fab), which contains variable regions coding for antigen recognition, and the constant fragment (Fc), which determines the function of the antibody. Based on the Fc region, immunoglobulins are designated IgA, IgD, IgE, IgG, or IgM. The cross-linking of antibody molecules on the surface of a cell leads to the activation of complement, resulting in the destruction of the target by lytic cells or in phagocytosis by macrophages.

Antibody-forming cell (AFC) assay: Also termed plaque-forming cell (PFC) assay. This assay measures the ability of animals to produce either IgM or IgG antibodies against a T-dependent or T-independent antigen following in vivo (or less frequently in vitro) immunization. Because of the involvement of multiple cellular and humoral elements in mounting an antibody response, the assay evaluates several immune parameters simultaneously. It is considered to be one of the most sensitive indicator systems for rodent immunotoxicology studies.

Antigen: A molecule that is the target of a specific immune reaction. Antigens are recognized in a cognate fashion by either immunoglobulins or the antigen receptor on the surface of T cells. Antigens are usually proteinaceous in nature.

Antigen-presenting cell (APC): Cells responsible for making antigens accessible to immune effector and regulatory cells. Following internalization and degradation of the antigen (e.g., by phagocytes), a fragment of the antigen molecule is presented on the APC cell surface in association with histocompatibility molecules. The resulting complex is subsequently recognized either by B cells via surface-bound Ig molecules or by T cells via the T cell antigen receptor (TCR). The induction of a specific immune response then proceeds. Representative APC includes macrophages, dendritic cells, and certain B cells.

Autoimmunity: Reaction of the immune system against the host organism. In the context of drug development, autoimmunity may take the form of escape from tolerance, as when a drug modifies a host antigen, which is subsequently seen as foreign. Drug-induced

autoimmunity may also result from bystander damage to host tissues from a drug-specific immune reaction.

B cell/B lymphocyte: Lymphocytes that recognize antigen via surface-bound Ig. B cells that have been exposed to cognate antigen subsequently proliferate and differentiate into plasma cells, which are responsible for producing specific antibody. B cells differentiate in the bone marrow in mammals and in an organ known as the bursa of Fabricius in birds.

Bioassay: A functional assay that depends on living cells as an indicator system. It may be performed either *in vivo* or *in vitro/ex vivo*.

Biologics/biotherapeutics: Biotechnology-derived pharmaceuticals (biopharmaceuticals) such as monoclonal antibodies.

Cell-mediated immunity (CMI): Antigen-specific reactivity mediated primarily by T cells. CMI may take the form of immunoregulatory activity (mediated by CD4+ helper T cells) or immune effector activity (mediated by CD8+ killer T cells). Other forms of direct cellular activity in host defense (e.g., NK cells and macrophages) are not antigen specific and are more accurately referred to as innate immunity.

Chemokine/chemotactic cytokines: Small chemoattractant proteins important in the stimulation and migration of immune cells. Chemokines are divided into four groups depending on the positions and spacing of the cysteine residues.

Cluster of differentiation (CD): A series of molecules expressed by immune cells on their cell surface. These proteins serve various physiological roles *in vivo*, usually as receptors or ligands. The CD nomenclature was standardized in 1982 and has been expanded since then as new molecules are characterized. The CD nomenclature is especially useful in immunophenotyping immune cell types based on the proteins present on their surface.

Complement: A group of approximately 20 protein precursor molecules assemble into a complex that intercalates into the membrane of a cell and forms a pore resulting in osmotic lysis of the target cell.

Cytokine: Small peptides produced primarily by cells of the immune system, particularly helper T cells. Cytokines are grouped into nonexclusive categories including interleukins, tumor necrosis factors, interferons, colony-stimulating factors, and various miscellaneous cytokines. Related molecules include peptide growth factors, transforming growth factors, and chemokines. Cytokines form an interactive network with both hormones and neuropeptides. Cytokines may be referred to in the older literature as lymphokines.

Cytotoxic T lymphocyte (CTL): A subset of CD8+ T cells able to kill target cells following the induction of a specific immune response. The mechanism of lysis appears to be a combination of direct lysis by extravasation of lytic molecules (such as perforins

and granzymes), as well as the induction of apoptosis in the target cell. Measurement of CTL activity is a sensitive indicator of CMI.

Delayed-type hypersensitivity (DTH): A form of CMI in which secondary exposure to an antigen results in an inflammatory reaction mediated by CD4+ T cells.

Enzyme-linked immunosorbent assay (ELISA): A type of immunoassay in which specific antibodies are used to capture and detect molecules of interest from a fluid matrix. The most common form is a "sandwich" ELISA in which the antibodies are bound to a substrate such as a plastic culture plate, and a second labeled antibody is used to detect the bound molecules.

Hapten: Low molecular weight molecules that are not antigenic by themselves but that are recognized as antigens when bound to larger molecules, usually proteins.

Host defense: The ability of an organism to protect itself against disease associated with exposure to infectious organisms, foreign tissue, or neoplasia. Host defense assays measure protection from infectious or neoplastic disease and are mediated by an immunological effect involving all components of the immune system—innate, cell-mediated, and humoral-mediated immunity (HMI). Host resistance assays are the only way to demonstrate immunological reserve.

Humoral-mediated immunity (HMI): Specific immune responses mediated primarily by humoral factors including antibodies and complement. The induction of HMI generally, although not exclusively, requires the cooperation of cellular immune mechanisms.

Hypersensitivity: A vigorous and often inappropriate immune response to seemingly innocuous antigens. Hypersensitivity is classified into subtypes depending on the mechanisms of action and the target cells or tissues.

Immunoassay: Refers to any assay that employs specific antibodies as reagents.

Immunological reserve: The concept that the immune response exhibits multiple immunological functions acting in an orchestrated manner, such that a decrease in one function is compensated by other immune functions. Immunological reserve prevents infectious or neoplastic disease due to acute reductions in one or two immune functions. This reserve would theoretically prevent a severe reduction in host resistance following a temporary immunosuppression of selected immune functions. This concept is important in interpretation of immunotoxicology data.

Immunostimulation: Enhancement of immune function above an accepted baseline (control) level. Immunostimulation may be beneficial, for example, therapeutics designed to restore a suboptimal immune response. It may conversely be detrimental, as would be the case with autoimmunity or hypersensitivity.

Immunosuppression: Depression/reduction of immune function below an accepted baseline (control) level. Immunosuppression may result from inadvertent exposure to drugs or other chemical or physical agents, intentional modification for therapeutic reasons (e.g., organ transplantation), or following exposure to certain infectious agents (e.g., HIV). An important consideration in immunotoxicology is the ability to determine the amount of immunosuppression necessary to alter host defense. Immunosuppression may result in a state of immunodeficiency.

Immunotoxicology: The discipline of synergistically applying cardinal principles of both immunology and toxicology to study the ability of certain treatments to alter the immune response in an adverse manner.

Inflammation: A nonspecific host defense mechanism characterized by the infiltration of leukocytes into the peripheral tissue, followed by the release of various mediators eliciting nonspecific physiological defense mechanisms. A normal prelude to a specific immune response; unchecked inflammation can result in extensive tissue damage.

Innate (natural or nonspecific) immunity: Host defense mechanisms that do not require prior exposure to antigen; often are antigen nonspecific in nature. Nonspecific immunity is mediated by NK cells, macrophages, neutrophils, $\gamma\delta$ T cells, and complement.

Lymphoproliferation: Proliferation of lymphocytes in response to stimulation with cellular activators such as antigens, mitogens, or allogeneic cells. Because proliferation is one of the initial consequences of activation, lymphoproliferation is used as a nonspecific indicator of immune responsiveness. This reaction is also referred to as “blastogenesis” or “mitogenesis.”

Macrophage: A bone marrow-derived cell present in the peripheral tissue that serves a wide variety of host defense functions, acting as both nonspecific phagocytes and killer cells, as well as regulators of specific immune reactions. Macrophages have different designations depending on the tissue in which they are located, such as Kupffer cells in the liver and veiled cells in the lymphatic system.

Major histocompatibility complex (MHC): Cell surface molecules that determine tissue compatibility and regulate self-recognition and tolerance. Two major classes are recognized: Class I (present on all nucleated cells) and Class II (present on B cells, T cells, and macrophages). MHC molecules direct the course of immune reactivity and are presented in association with antigens by antigen presenting cells. In humans, MHCs are specifically referred to as human leukocyte antigen (HLA).

Mitogen: Molecules capable of inducing cellular activation; these may be protein or polysaccharide in nature.

Mixed lymphocyte response/reaction (MLR): An in vitro assay that measures the ability of lymphocytes to respond to the presence of allogeneic cells. This proliferation represents the initial stage of the acquisition of cytotoxic T lymphocytes function by CD8⁺ T cells and thus serves as a measure of cell-mediated immunity. The MLR is a form of lymphoproliferation. Also referred to as mixed lymphocyte culture (MLC).

Mononuclear phagocyte system: Previously known as the reticuloendothelial system (RES), this system is composed of all phagocytic cells of the body, including monocytes/macrophages and polymorphonuclear cells (i.e., neutrophils).

Mucosa-associated lymphoid tissue (MALT): Lymphoid cells and tissues lining the mucosa that serve as the first point of contact with antigen encountered via this route. MALT comprises Peyer's patches, the appendix, tonsils, and lymphoid cells in the lamina propria of the gut.

Natural killer (NK) cells: A population of lymphocytes distinct from T and B cells, also referred to as large granular lymphocytes (LGLs) because of their microscopic appearance. NK cells exhibit cytotoxicity against virally infected cells and certain tumor cells. Assessment of NK cell function provides a good measure of innate immunity.

New molecular entity (NME): A novel molecule under development for pharmaceutical purposes. The term encompasses both new chemical entities (NCEs), such as small molecule drugs, and new biological entities (NBEs).

Peripheral blood mononuclear cells (PBMCs)/peripheral blood mononuclear leukocytes (PBMLs): Leukocytes derived from the peripheral circulation. Because of their accessibility, these cells are often used in *ex vivo* immune function assessment.

Skin immune system (SIS): Cells associated with the skin that participate in immunity. Includes Langerhans cells, dendritic cells, and keratinocytes. Alternatively known as skin-associated lymphoid tissue (SALT).

T cell/T lymphocyte: Lymphocytes that recognize specific antigens via a complex of molecules known collectively as the T cell antigen receptor. T cells are primarily responsible for the induction and maintenance of cell-mediated immunity, although they also regulate humoral-mediated immunity and some nonimmune effector mechanisms. A variety of T cell subpopulations exist, including helper T cells, cytotoxic T cells, inducer T cells, and regulatory T cells. T cells mature in the thymus.

Xenobiotic: Any substance that is foreign to an organism. In the context of immunotoxicology, the term generally refers to nonbiological chemicals or drugs.

MEDICAL DEVICES

Biomaterial: A material that has direct or indirect patient contact. A biomaterial (also termed a *biomedical material*) may be composed of any synthetic or

natural rubber or fiber, polymeric or elastomeric formulation, alloy, ceramic, bonding agent, ink, or other nonviable substance, including tissue rendered nonviable, used as a device or any part thereof.

Class testing: The testing of plastics for biological reactivity according to predetermined testing requirements defined by the US Pharmacopeia (USP).

Combination (medical device) product: A product containing both a drug and a device component that are physically, chemically, or otherwise combined to result in a medical product that is used therapeutically as a single entity. The medical device component must be evaluated for safety according to device requirements, the drug component must be evaluated for safety as per drug requirements, and the safety of the finished combined product must be also be evaluated.

Direct contact: When the materials of a medical device are in direct (i.e., intimate) contact with the surface or tissues of the body (e.g., adhesive bandages, pace-maker leads).

Extract: A solution produced by the incubation of a material/medical device in an appropriate vehicle. After incubation, the vehicle contains the soluble chemicals (or leachables) that have dissolved out of, or off, the material/medical device.

Indirect contact: When materials of a medical device do not contact the surface or tissues of the body, the materials of the device may influence the body. In this case, a solution or other material that contacts the device may become contaminated with leachables from the device that in turn contacts tissues of the body (e.g., intravenous infusion bag).

ISO: International Standards Organization.

Medical device: Any instrument, apparatus, appliance, material, or other articles, including software, whether used alone or in combination, intended by the manufacturer for use by human beings solely or principally for the purpose of diagnosis, prevention, monitoring, or treatment; alleviation of disease, injury, or handicap; investigation, replacement, or modification of the anatomy or of a physiological process; control of conception; and that which does not achieve its principal intended action of the body by pharmaceutical, immunological, or metabolic means but may be assisted in its function by such means.

Predicate device: A previously marketed medical device that is substantially equivalent to a proposed device. The predicate device is used as a comparison to the proposed device to establish safety and efficacy.

Processing aid: A material that contacts a medical device product during the manufacturing process and, therefore, has a potential for affecting product quality and/or may elicit a biological response following the use of a medical device. Solvents, cleaning products, lubricants, and mold-release agents are examples of processing aids.

USP negative control plastic RS: A standardized plastic produced by the USP for use as a control material in some biocompatibility assays.

NEUROTOXICOLOGY

Akinesia: Absence or the loss of power of voluntary motion; immobility.

Ataxia: Incoordination; the inability to coordinate the muscles in the execution of voluntary movement.

Catalepsy: Condition in which there is waxy rigidity of the limbs that may be placed in various positions that will be maintained for a time.

Clonic convulsion: A convulsion in which the muscles alternately relax and contract.

Clonus: A form of movement characterized by contractions and relaxations of a muscle.

Convulsion: A violent spasm of the face, trunk, or extremities.

Dysarthria: Disturbance of articulation due to emotional stress or to paralysis, incoordination, or spasticity of muscles used in vocalizing.

Dyskinesia: Difficulty in performing voluntary movements; a movement disorder characterized by insuppressible, stereotyped, and automatic movements.

Dystonia: Abnormal tonic (hyper or hypo) in any tissues.

Fasciculations: Involuntary contractions, or twitching, of groups of muscle fibers.

Hyperkinesia: Excessive muscular activity.

Myoclonus: Brief, involuntary twitching of a muscle or a group of muscles.

Myotonia: Delayed relaxation of a muscle after an initial contraction.

Paresthesia: An abnormal sensation, such as burning, prickling, tickling, or tingling.

Stereotypy: The constant repetition of gestures or movements that appear to be excessive or purposeless.

Tonic convulsion: A convulsion in which muscle contraction is sustained.

OCULAR TOXICOLOGY

Anterior chamber: The aqueous-containing cavity of the eye, bounded by the cornea anteriorly, the chamber angle structures peripherally, and the iris and lens posteriorly.

Blepharitis: Inflammation of the eyelids.

Blepharospasm: Involuntary spasm of the lids.

Cataract: An opacity of the lens or its capsule.

Chemosis: Intense edema of the conjunctiva. The conjunctiva is loose fibrovascular connective tissue that is relatively rich in lymphatics and responds to noxious stimuli by swelling to the point of prolapse between the lids.

Choroid: The vascular middle coat between the retina and sclera.

Ciliary body: Portion of the uveal tract between the iris and the choroid consisting of ciliary processes and the ciliary muscle.

Conjunctiva: Mucous membrane that lines the posterior aspect of the eyelids (palpebral conjunctiva) and the anterior sclera (bulbar conjunctiva).

Conjunctivitis: Inflammation of the conjunctiva.

Cornea: Transparent portion of the outer coat of the eyeball forming the anterior wall of the anterior chamber.

Exophthalmos: Abnormal protrusion of the eyeball.

Fluorescein (fluorescein sodium): A fluorescent dye, the simplest of the fluorane dyes and the mother substance of eosin, which is commonly used intravenously to determine the state of adequacy of circulation in the retina and a lesser degree the choroid and iris. Another important use is to detect epithelial lesions of the cornea and conjunctiva. Peak excitation occurs with light at a wavelength between 485 and 500 μm , and peak emission occurs between 520 and 530 μm .

Fovea: Depression in the macula adapted for most acute vision.

Fundus: The posterior portion of the eye visible through an ophthalmoscope.

Hyperemia: Excess of blood in a part due to local or general relaxation of the arterioles. Blood vessels become congested and give the area involved a reddish or red-blue color.

Injection: Congestion of blood vessels.

Iris: The circular pigmented membrane behind the cornea and immediately in front of the lens; the most anterior portion of the vascular tunic of the eye. It is composed of the dilator and sphincter muscles and the two-layered posterior epithelium and mesodermal components that form the iris stroma.

Iritis: Inflammation of the iris, manifested by vascular congestion (hyperemia). An outpouring of serum proteins into the aqueous (flare) may accompany the inflammatory reaction.

Keratitis: Inflammation of the cornea.

Lens: A transparent biconvex structure suspended in the eyeball between the aqueous and the vitreous. Its function is to bring rays of light to a focus on retina. Accommodation is produced by variations in the magnitude of this effect.

Miotic: A drug causing pupillary constriction.

Mydriatic: A drug causing pupillary dilatation.

Nystagmus: An involuntary, rapid movement of the eyeball that may be horizontal, vertical, rotatory, or mixed.

Optic disk: Ophthalmoscopically visible portion of the optic nerve.

Palpebral: Pertaining to the eyelid.

Pannus: Vascularization and connective-tissue deposition beneath the epithelium of the cornea.

Posterior chamber: Space filled with aqueous anterior to the lens and posterior to the iris.

Ptosis: Drooping of the upper eyelid.

Pupil: The round opening at the center of the iris that allows transmission of light to the posterior of the eyeball.

Retina: The innermost or nervous tunic of the eye that is derived from the optic cup (the outer layer develops into the complex sensory layer).

Sclera: The white tough covering of the eye that, with the cornea, forms the external protective coat of the eye.

Vitreous: Transparent, colorless, mass of soft, gelatinous material filling the space in the eyeball posterior to the lens and anterior to the retina.

PHARMACEUTICALS

Abbreviated new drug application (ANDA): An ANDA contains data that, when submitted to FDA's Center for Drug Evaluation and Research, Office of Generic Drugs, provide for the review and ultimate approval of a generic drug product. Generic drug applications are called "abbreviated" because they are generally not required to include preclinical (animal) and clinical (human) data to establish safety and effectiveness. Instead, a generic applicant must scientifically demonstrate that its product is bioequivalent (i.e., performs in the same manner as the innovator drug). Once approved, an applicant may manufacture and market the generic drug product to provide a safe, effective, low-cost alternative.

Active ingredient: Any component that provides pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or affects the structure or any function of the body of man or animals.

ADME: Absorption, distribution, metabolism, and excretion.

Biological license application (BLA): Biological products are approved for marketing under the provisions of the Public Health Service (PHS) Act. The Act requires a firm who manufactures a biologic for sale in interstate commerce to hold a license for the product. A biologics license application is a submission that contains specific information on the manufacturing processes, chemistry, pharmacology, clinical pharmacology, and the medical effects of the biological product. If the information provided meets FDA requirements, the application is approved, and a license is issued allowing the firm to market the product.

Biological product: A biological product is any virus, serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries. Biological products are a subset of "drug products" distinguished by their manufacturing processes (biological process vs. chemical process). In general, the term "drugs" includes biological products.

CBER: FDA's Center for Biologics Evaluation and Research—the division charged with regulating biological products.

CDER: FDA's Center for Drug Evaluation and Research—the division charged with developing and enforcing policy with regard to the safety, effectiveness, and labeling of all drug products for human use.

Drug: A substance recognized by an official pharmacopoeia or formulary. A substance intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease. A substance (other than food) intended to affect the structure or any function of the body. A substance intended for use as a component of a medicine but not a device or a component, part, or accessory of a device. Biological products are included within this definition and are generally covered by the same laws and regulations, but differences exist regarding their manufacturing processes (chemical process vs. biological process).

Human equivalent dose (HED): A dose in humans anticipated to provide the same degree of effect as that observed in animals at a given dose. In drug development, the term HED is usually used to refer to the HED of the no-observed-adverse-effect level (NOAEL).

Identification threshold: A limit (%) above which an impurity or degradation product should be identified.

IND: Investigational New Drug Application—a request to initiate a clinical study of a new drug product.

Label: The FDA-approved label is the official description of a drug product that includes indication (what the drug is used for); who should take it; adverse events (side effects); instructions for uses in pregnancy, children, and other populations; and safety information for the patient. Labels are often found inside drug product packaging.

Maximum recommended starting dose (MRSD): The highest dose recommended as the initial dose of a drug in a clinical trial. The MRSD is predicted to cause no adverse reactions.

New drug application (NDA): When the sponsor of a new drug believes that enough evidence on the drug's safety and effectiveness has been obtained to meet FDA's requirements for marketing approval, the sponsor submits to FDA an NDA. The application must contain data from specific technical viewpoints for review, including chemistry, pharmacology, medical, biopharmaceutics, and statistics. If the NDA is approved, the product may be marketed in the United States. For internal tracking purposes, all NDAs are assigned an NDA number.

Pharmacologically active dose (PAD): The lowest dose tested in an animal species with the intended pharmacological activity.

PLA: Product License Application for a biologic.

Qualification: For pharmaceuticals, the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or degradation product or a given impurity or degradation profile at the level(s) specified.

Qualification threshold: A limit (%) above which an impurity or degradation product should be qualified.

Reporting threshold: A limit (%) above which an impurity or degradation product should be reported.

REPRODUCTIVE/DEVELOPMENTAL TOXICOLOGY

(See Chapter 10 for terms arranged by body part/organ system.)

Aberration: A minor structural change. It may be a retardation (a provisional delay in morphogenesis), a variation (external appearance controlled by genetic and extragenetic factors), or a deviation (resulting from altered differentiation).

Ablepharia: Absence or reduction of the eyelid(s).

Abrachius: Without arms, forelimbs.

Acardia: Absence of the heart.

Acaudia (anury): Agenesis of the tail.

Accessory spleen: An additional spleen.

Acephaly: Congenital absence of the head.

Achondroplasia: A hereditary defect in the formation of epiphyseal cartilage, resulting in a form of dwarfism with short limbs, normal trunk, small face, normal vault, etc.

Acrania: Partial or complete absence of the skull.

Acystia: Absence of the urinary bladder.

Adactyly: Absence of digits.

Agastria: Absence of the stomach.

Agenesis: Absence of an organ or part of an organ.

Agenesis of the kidney: Absence of the kidney(s).

Agenesis of the lung (lobe): Complete absence of a lobe of the lung.

Aglossia: Absence of the tongue.

Agathia: Absence of lower jaw (mandible).

Anal atresia: Congenital absence of the anus.

Anencephaly: Congenital absence of the cranial vault with missing or small brain mass.

Anomaly or abnormality: A morphological or functional deviation from normal limit. It can be a malformation or a variation.

Anophthalmia: Absence of eye(s).

Anorchism: Congenital absence of one or both testes.

Anotia: Absence of the external ear(s).

Aphalangia: Absence of a finger or a toe; corresponding metacarpals not affected.

Aplasia: Lack of development of an organ, frequently used to designate complete suppression or failure of development of a structure from the embryonic primordium.

Aplasia of the lung: The trachea shows rudimentary bronchi, but pulmonary and vascular structures are absent.

Apodia: Absence of one or both feet.

Aproctia: Imperforation or absence of anus.

- Arrhinia:** Absence of nose.
- Arthrogryposis:** Persistent flexure or contracture of a joint; flexed paw (bent at wrist) is the most common form of arthrogryposis.
- Aspermia:** No ejaculate
- Astomia:** Absence of oral orifice.
- Asthenozoospermia:** More than 50% spermatozoa with poor (<2 grade) forward progression.
- Azoospermia:** No spermatozoa in the ejaculate.
- Brachydactyly:** Shortened digits.
- Brachyury (short tail):** Tail that is reduced in length.
- Bulbous rib:** Having a bulge or balloon-like enlargement somewhere along its length.
- Cardiomegaly:** Hypertrophy (enlargement) of the heart.
- Cardiovascular situs inversus:** Mirror-image transposition of the heart and vessels to the other side of the body.
- Cephalocele:** A protrusion of a part of the cranial contents, not necessarily neural tissue.
- Conceptus:** The sum of derivatives of a fertilized ovum at any stage of development from fertilization until birth. Also term used when the stage of prenatal development at the time of initial insult is not known; also referred to as *embryo–fetus*.
- Corpus luteum:** The yellow endocrine body formed in the ovary at the site of the ruptured Graafian follicle.
- Craniorachischisis:** Exencephaly and holorrhachischisis (fissure of the spinal cord).
- Cranioschisis:** Abnormal fissure of the cranium; may be associated with meningocele or encephalocele.
- Cryptorchidism (undescended testes, ectopic testes):** Failure of the testes to descend into the scrotum (can be unilateral).
- Cyclopia:** One central orbital fossa with none, one, or two globes.
- Deflection:** A turning, or state of being turned, aside.
- Deformity:** Distortion of any part or general disfigurement of the body.
- Deviation:** Variation from the regular standard or course.
- Dextrogastria:** Having the stomach on the right side of the body.
- Dextrocardia:** Location of the heart in the right side of the thorax; a developmental disorder that is associated with total or partial situs inversus (transposition of the great vessels and other thoracoabdominal organs) or occurs as an isolated anomaly.
- Displaced rib:** Out of normal position.
- Dysgenesis:** Defective development; malformation.
- Dysmelia:** Absence of a portion of one or several limbs.
- Dysplasia:** (a) Abnormal development of tissues; (b) alteration in size, shape, or organization of adult cells.
- Dystocia:** Abnormal labor.
- Ectocardia:** Displacement of the heart inside or outside the thorax.
- Ectopic:** Out of the normal place.
- Ectopic esophagus:** Displacement of the esophagus (description of position should be included).
- Ectopic pinna:** Displaced external ear.
- Ectrodactyly:** Absence of all or of only a part of digit (partial ectrodactyly).
- Ectromelia:** Aplasia or hypoplasia of one or more bones of one or more limbs (this term includes amelia, hemimelia, and phocomelia).
- Encephalocele:** A partial protrusion of brain through an abnormal cranial opening; not as severe as exencephaly.
- Estrus:** Phase of the sexual cycle of female mammals characterized by willingness to mate.
- Exencephaly:** Brain outside of the skull as a result of a large cranial defect.
- Exomphalos:** Congenital herniation of abdominal viscera into umbilical cord.
- Exophthalmos:** Protrusion of the eyeball (“pop” eye).
- Fecundity:** Ability to produce offspring rapidly and in large numbers.
- Fetocide:** The destruction of the fetus in the uterus.
- Gamete:** A male (spermatozoa) or female (ovum) reproductive cell.
- Gastroschisis:** Fissure of the abdominal wall (median line) not involving the umbilicus, usually accompanied by protrusion of the small part of the large intestine, not covered by membranous sac.
- Hemivertebra:** Presence of only one-half of a vertebral body.
- Hepatic lobe agenesis:** Absence of a lobe of the liver.
- Hepatomegaly:** Abnormal enlargement of the liver.
- Hydrocephaly:** Enlargement of the head caused by abnormal accumulation of cerebrospinal fluid in subarachnoid cavity (external hydrocephaly) or ventricular system (internal hydrocephaly).
- Hydronephrosis:** Dilatation of the renal pelvis usually combined with destruction of renal parenchyma and often with dilation of the ureters (bilateral, unilateral). Note: This is a pathology term and should have histological confirmation.
- Hypoplasia of the lung:** Bronchial tree is poorly developed, and pulmonary tissue shows an abnormal histological picture (total or partial); incomplete development, smaller.
- Hypospadias:** Urethra opening on the underside of the penis or on the perineum (males) or into the vagina (females).
- Imperforate:** Not open; abnormally closed.
- Incomplete ossification (delayed, retarded):** The extent of ossification is less than what would be expected for that developmental age, not necessarily associated with reduced fetal or pup weight.
- Levocardia:** Displacement of the heart in the extreme left hemithorax.
- Lordosis:** Anterior concavity in the curvature of the cervical and lumbar spine as viewed from the side.
- Macrobrachia:** Abnormal size or length of the arm.
- Macroactylia:** Excessive size of one or more digits.
- Macroglossia:** Enlarged tongue, usually protruding.
- Macrophthalmia:** Enlarged eye(s).

Malformation: Defective or abnormal formation; deformity. A permanent structural deviation that generally is incompatible with, or severely detrimental to, normal survival or development.

Meiosis: Cell division occurring in maturation of the sex cell (gametes) by means of which each daughter nucleus receives half the number of chromosomes characteristic of the somatic cells of the species.

Microcephaly: Small head.

Micrognathia: Shortened lower jaw (mandible); tongue may protrude.

Microphthalmia: Small eyes.

Microstomia: Small mouth opening.

Microtia: Small external ear.

Monocardium: Possessing a heart with only one atrium and one ventricle.

Multigravida: A female pregnant for the second (or more) time.

Naris (nostril) atresia: Absence or closure of nares.

Nasal agenesis: Absence of the nasal cavity and external nose.

Normozoospermia: Normal semen sample.

Nulliparous: A female that has never borne a viable offspring.

Oligodactyly: Fewer than normal number of digits.

Oligohydramnios (oligoamnios): Reduction in the amount of amniotic fluid.

Oligozoospermia: A subnormal sperm concentration in ejaculate.

Omphalocele: Midline defect in the abdominal wall at the umbilicus, through which the intestines and often other viscera (stomach, spleen, and portions of the liver) protrude. These are always covered by a membranous sac. As a rule, the umbilical cord emerges from the top of the sac.

Pachynsis: Abnormal thickening.

Patent ductus arteriosus (ductus botalli): An open channel of communication between the main pulmonary artery and the aorta; may occur as an isolated abnormality or in combination with other heart defects.

Polydactyly: Extra digits.

Polysomia: A fetal malformation consisting of two or more imperfect and partially fused bodies.

Pseudopregnancy: (a) False pregnancy: condition occurring in animals in which anatomical and physiological changes occur similar to those of pregnancy; (b) the premenstrual stage of the endometrium: so called because it resembles the endometrium just before the implantation of the blastocyst.

Rachischisis: Absence of vertebral arches in limited area (partial rachischisis) or entirely (rachischisis totalis).

Renal hypoplasia: Incomplete development of the kidney.

Resorption: A conceptus that, having implanted in the uterus, subsequently died and is being or has been resorbed.

Rhinocephaly: A developmental anomaly characterized by the presence of a proboscis-like nose above the eyes, partially or completely fused into one.

Rudimentary rib: Imperfectly developed riblike structure.

Schistoglossia: Cleft tongue.

Seminiferous epithelium: The normal cellular components within the seminiferous tubule consisting of Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids.

Septal agenesis: Absence of nasal septum.

Sertoli cells: Cells in the testicular tubules providing support, protection, and nutrition for the spermatids.

Spermatocytogenesis: The first stage of spermatogenesis in which spermatogonia develop into spermatocytes and then into spermatids.

Spermiation: The second stage of spermatogenesis in which the spermatids transform into spermatozoa.

Spina bifida: Defect in closure of bony spinal cavity.

Symphodia: Fusion of the lower extremities.

Syndactyly: Partially or entirely fused digits.

Tetralogy of Fallot: An abnormality of the heart that includes pulmonary stenosis, ventricular septal defect, dextroposition of the aorta overriding the ventricular septum and receiving blood from both ventricles, and right ventricular hypertrophy.

Teratozoospermia: Fewer than 30% spermatozoa with normal morphology.

Thoracogastroschisis: Midline fissure in the thorax and abdomen.

Totalis or partialis: Total or partial transposition of viscera (due to incomplete rotation) to the other side of the body; heart most commonly affected (dextrocardia).

Tracheal stenosis: Constriction or narrowing of the tracheal lumen.

Unilobular lung: In the rat fetus, a condition in which the right lung consists of one lobe instead of four separate lobes.

Vaginal plug: A mass of coagulated semen that forms in the vagina of animals after coitus; also called copulation plug or bouchon vaginal.

Variation: An alteration that represents a retardation in development, a transitory alteration, or a permanent alteration not believed to adversely affect survival, growth, development, or functional competence.

RISK ASSESSMENT/GENERAL TOXICOLOGY

Absorbed dose: The amount of a substance penetrating across the exchange boundaries of an organism and into body fluids and tissues after exposure.

Acceptable daily intake (ADI): A value used for noncarcinogenic effects that represents a daily dose that is very likely to be safe over an extended period of time. An ADI is similar to a reference dose (RfD) (defined in the succeeding text) but less strictly defined.

Administered dose: The amount of a substance given to a human or test animal in determining dose-response relationships, especially through ingestion or inhalation (see Applied dose). Administered dose

is actually a measure of exposure, because even though the substance is “inside” the organism, once ingested or inhaled, administered dose does not account for absorption (see Absorbed dose).

Aggregate risk: The sum of individual increased risks of an adverse health effect in an exposed population.

Applied dose: The amount of a substance given to a human or test animal in determining dose–response relationships, especially through dermal contact. Applied dose is actually a measure of exposure, since it does not take absorption into account (see Absorbed dose).

Biological significant effect: A response in an organism or other biological systems that is considered to have a substantial or noteworthy effect (positive or negative) on the well-being of the biological system. Used to distinguish from statistically significant effects or changes, which may or may not be meaningful to the general state of health of the system.

Cancer potency factor (CPF): The statistical 95% upper confidence limit on the slope of the dose–response relationship at low doses for a carcinogen. Values are in units of lifetime risk per unit dose (mg/kg/day). A plausible upper bound risk is derived by multiplying the extended lifetime average daily dose (LADD) by the CPF.

Case-control study: A retrospective epidemiologic study in which individuals with the disease under study (cases) are compared with individuals without the disease (controls) in order to contrast the extent of exposure in the diseased group with the extent of exposure in the controls.

Ceiling limit: A concentration limit in the work place that should not be exceeded, even for a short time, to protect workers against frank health effects.

CFR: Code of Federal Regulations (United States).

Cohort study: A study of a group of persons sharing a common experience (e.g., exposure to a substance) within a defined time period; the experiment is used to determine if an increased risk of a health effect (disease) is associated with that exposure.

Confidence limit: The confidence interval is a range of values that has a specified probability (e.g., 95%) of containing a given parameter or characteristic. The confidence limit often refers to the upper value of the range (e.g., upper confidence limit).

Critical endpoint: A chemical may elicit more than one toxic effect (endpoint), even in one test animal, in tests of the same or different duration (acute, subchronic, and chronic exposure studies). The doses that cause these effects may differ. The critical endpoint used in the dose–response assessment is the one that occurs at the lowest dose. In the event that data from multiple species are available, it is often the most sensitive species that determines the critical endpoint. This term is applied in the derivation of risk reference doses (RfDs).

Cross-sectional study: An epidemiologic study assessing the prevalence of a disease in a population. These studies are most useful for conditions or diseases that are not expected to have a long latent period and do not cause death or withdrawal from the study population. Potential bias in case ascertainment and exposure duration must be addressed when considering cross-sectional studies.

De minimus risk: From the legal maxim, “*de minimus non curat lex*” or the law is not concerned with trifles. As relates to risk assessment of carcinogens, it is commonly interpreted to mean that a lifetime risk of 1×10^{-6} is a *de minimus* level of cancer risk (i.e., insignificant and therefore acceptable) and is of no public health consequence.

Dispersion model: A mathematical model or computer simulation used to predict the movement of airborne or water airborne contaminants. Models take into account a variety of mixing mechanisms that dilute effluents and transport them away from the point of emission.

Dose: The amount of substance administered to an animal or human generally expressed as the weight or volume of the substance per unit of body weight (e.g., mg/kg, mL/kg).

Dose–response relationship: A relationship between the dose, often actually based on “administered dose” (i.e., exposure) rather than absorbed dose, and the extent of toxic injury produced by that chemical. Response can be expressed as either the severity of injury or proportion of exposed subjects affected. A dose–response assessment is one of the steps in a risk assessment.

Duration of exposure: Generally referred to in toxicology as acute (one time), subacute (repeated over several weeks), subchronic (repeated for a fraction of a lifetime), and chronic (repeated for nearly a lifetime).

Endemic: Present in a community or among a group of people; said of a disease prevailing continually in a region.

Environmental fate: The destiny of a chemical or biological pollutant after release into the environment. Environmental fate involves temporal and spatial considerations of transport, transfer, storage, and transformation.

Exposure: Contact of an organism with a chemical, physical, or biological agent. Exposure is quantified as the amount of the agent available at the exchange boundaries of the organism (e.g., skin, lungs, digestive tract) and available for absorption.

Exposure frequency: The number of times an exposure occurs in a given period. The exposure(s) may be continuous, discontinuous but regular (e.g., once daily), or intermittent.

Extrapolation: An estimate of response or quantity at a point outside the range of the experimental data. Also refers to the estimation of a measured response

in a different species or by a different route than that used in the experimental study of interest (i.e., species to species, route to route, acute to chronic, high to low).

Fence line concentration: Modeled or measured concentrations of pollutants found at the boundaries of a property on which a pollution source is located. Usually assumed to be the nearest location at which an exposure of the general population could occur.

Frank effect level (FEL): Related to biological responses to chemical exposures (compare with NOAEL and LOEL); the exposure level that produces an unmistakable adverse health effect (such as inflammation, severe convulsions, or death).

Hazard: The inherent ability of a substance to cause an adverse effect under defined conditions of exposure.

Hazard index: The ratio of the maximum daily dose (MDD) to the ADI used to evaluate the risk of noncarcinogens. A value of less than 1 indicates that the risk from the exposure is likely insignificant; a value greater than 1 indicates a potentially significant risk.

HED: The human dose of an agent expected to induce the same type and severity of toxic effect that an animal dose has induced.

Immediately dangerous to life and health (IDLH): A concentration representing the maximum level of a pollutant from which an individual could escape within 30 min without escape-impairing symptoms or irreversible health effects.

Incidence: The number of new cases of a disease within a specified time period. It is frequently presented as the number of new cases per 1,000, 10,000, or 100,000. The incidence rate is a direct estimate of the probability or risk of developing a disease during a specified time period.

Involuntary risk: A risk that impinges on an individual without their awareness or consent.

Latency: The period of time between exposure to an injurious agent and the manifestation of a response.

LC_{LO} (lethal concentration low): The lowest concentration of a chemical required to cause death in some of the population after exposure for a specified period of time and observed for a specified period of time after exposure. Refers to inhalation time exposure in the context of air toxics (may refer to water concentration for tests of aquatic organisms).

LC₅₀ (median lethal concentration): The concentration of a chemical required to cause death in 50% of the exposed population when exposed for a specified time period and observed for a specified period of time after exposure. Refers to inhalation exposure concentration in the context of air toxics (may refer to water concentration for tests of aquatic organisms).

LD_{LO} (lethal dose low): The lowest dose of a chemical required to cause death in some of the population after non-inhalation exposure (e.g., injection, ingestion), for a specified observation period after exposure.

LD₅₀ (median lethal dose): The dose of a chemical required to cause death in 50% of the exposed population after noninhalation exposure (e.g., injection, ingestion), for a specified observation period after exposure.

LADD: The total dose received over a lifetime multiplied by the fraction of a lifetime during which exposure occurs, expressed in mg/kg body weight/day.

Lifetime risk: A risk that results from lifetime exposure.

Lowest-observed-adverse-effect level (LOAEL): The lowest dose or exposure level of a chemical in a study at which there is a statistically or biologically significant increase in the frequency or severity of an *adverse* effect in the exposed population as compared with an appropriate, unexposed control group.

Lowest-observed-effect level (LOEL): In a study, the lowest dose or exposure level of a chemical at which a statistically or biologically significant effect is observed in the exposed population compared with an appropriate unexposed control group. The effect is generally considered not to have an adverse effect on the health and survival of the animal. This term is occasionally misused in place of a LOAEL.

Margin of exposure (MOE): The ratio of the NOAEL to the estimated human exposure. The MOE was formerly referred to as the margin of safety (MOS).

Maximum contaminant level (MCL): The maximum level of a contaminant permissible in water as defined by regulations promulgated under the Safe Drinking Water Act.

Maximum daily dose (MDD): Maximum dose received on any given day during a period of exposure generally expressed in mg/kg body weight/day.

Maximum tolerated dose (MTD): The highest dose of a toxicant that causes toxic effects without causing death during a chronic exposure and that does not decrease the body weight by more than 10%.

Modifying factor (MF): A factor that is greater than zero and less than or equal to 10; used in the operational derivation of an RfD. Its magnitude depends upon an assessment of the scientific uncertainties of the toxicological database not explicitly treated with standard uncertainty factor (UF) (e.g., the completeness of the overall database). The default value for the MF is 1. The use of an MF was discontinued by the EPA in 2004.

Multistage model: A mathematical function used to extrapolate the probability of incidence of disease from a bioassay in animals using high doses, to that expected to be observed at the low doses that are likely to be found in chronic human exposure. This model is commonly used in quantitative carcinogenic risk assessments where the chemical agent is assumed to be a complete carcinogen and the risk is assumed to be proportional to the dose in the low region.

Nonthreshold toxicant: An agent considered to produce a toxic effect from any dose; any level of exposure is deemed to involve some risk. Usually only in regard to carcinogenesis.

No-observed-adverse-effect level (NOAEL): The highest experimental dose at which there are not statistically or biologically significant increases in frequency or severity of *adverse* health effects, as seen in the exposed population compared with an appropriate, unexposed population. Effects may be produced at this level, but they are not considered to be adverse.

No-observed-effect level (NOEL): The highest experimental dose at which there is not statistically or biologically significant increase in the frequency or severity of effects seen in the exposed compared with an appropriate unexposed population.

Occupational exposure limit (OEL): A generic term denoting a variety of values and standards, generally time-weighted average (TWA) concentrations of airborne substances to which a worker can be safely exposed during defined work periods.

Permissible exposure limit (PEL): Similar to an OEL.

Potency: A comparative expression of chemical or drug activity measured in terms of the relationship between the incidence or intensity of a particular effect and the associated dose of a chemical, to a given or implied standard of reference. Can be used for ranking the toxicity of chemicals.

ppb: Parts per billion.

ppm: Parts per million.

Prevalence: The percentage of a population that is affected with a particular disease at a given time.

q1*: The symbol used to denote the 95% upper bound estimate of the linearized slope of the dose–response curve in the low-dose region as determined by the multistage model.

Reference dose (RfD): An estimate (with uncertainty spanning perhaps an order of magnitude or more) of the daily exposure to the human population (including sensitive subpopulations) that is likely to be without deleterious effects during a lifetime. The RfD is reported in units of mg of substance/kg body weight/day for oral exposures or mg/substance/m³ of air breathed for inhalation exposures (RfC).

Risk: The probability that an adverse effect will occur under a particular condition of exposure.

Risk assessment: The scientific activity of evaluating the toxic properties of a chemical and the conditions of human exposure to it in order both to ascertain the likelihood that exposed humans will be adversely affected and to characterize the nature of the effects they may experience. May contain some or all of the following four steps:

Hazard identification: The determination of whether a particular chemical is or is not causally linked to particular health effect(s)

Dose–response assessment: The determination of the relation between the magnitude of exposure and the probability of occurrence of the health effects in question

Exposure assessment: The determination of the extent of human exposure

Risk characterization: The description of the nature and often the magnitude of human risk, including attendant uncertainty

Risk management: The decision-making process that uses the results of risk assessment to produce a decision about environmental action. Risk management includes the consideration of technical, scientific, social, economic, and political information.

Short-term exposure limit (STEL): A TWA occupational exposure level (OEL) that the American conference of Government and Industrial Hygienists (ACGIH) indicates should not be exceeded any time during the work day. Exposures at the STEL should not be longer than 15 min and should not be repeated more than four times per day. There should be at least 60 min between successive exposure at the STEL.

Slope factor: See Cancer potency factor (CPF).

SNUR: Significant new use rule.

Standardized mortality ratio: The number of deaths, either total or cause specific, in a given group expressed as a percentage of the number of deaths that could have been expected if the group has the same age- and sex-specific rates as the general population. Used in epidemiologic studies to adjust mortality rates to a common standard so that comparisons can be made among groups.

STEL: See Short-term exposure limit (STEL).

Surface area scaling factor: The intra- and interspecies scaling factor most commonly used for cancer risk assessment by the US EPA to convert an animal dose to an HED: milligrams per square meter surface area per day. Body surface area is proportional to basal metabolic rate; the ratio of surface area to metabolic rate tends to be constant from one species to another. Since body surface area is approximately proportional to an animal's body weight to the 2/3 power, the scaling factor can be reduced to milligrams per body weight^{2/3}.

TC_{LO} (toxic concentration low): The lowest concentration of a substance in air required to cause a toxic effect in some of the exposed population.

TD_{LO} (toxic dose low): The lowest dose of a substance required to cause a toxic effect in some of the exposed population.

Threshold limit value (TLV): The TWA concentration of a substance below which no adverse health effects are expected to occur for workers assuming exposure for 8 h per day, 40 h per week. TLVs are published by the American Conference of Governmental Industrial Hygienists (ACGIH).

TWA: An approach to calculating the average exposure over a specified time period.

UF: One of several, generally 10-fold factors, applied to a NOAEL or a LOAEL to derive an RfD from experimental data. UFs are intended to account for (a) the variation in the sensitivity among the members of the human population, (b) the uncertainty in extrapolating animal data to human, (c) the uncertainty in extrapolating from data obtained in a less-than-lifetime exposure study to chronic exposure, (d) the uncertainty in using an LOAEL rather than an NOAEL for estimating the threshold region, and (e) uncertainty with extrapolation when the database is incomplete.

Unit cancer risk: A measure of the probability of an individual's developing cancer as a result of exposure to a specified unit ambient concentration. For example, an inhalation unit cancer risk of 3.0×10^{-4} near a point source implies that if 10,000 people breathe a given concentration of a carcinogenic agent (e.g., $1 \mu\text{g}/\text{m}^3$) for 70 years, three of the 10,000 will develop cancer as a result of this exposure. In water, the exposure unit is usually $1 \mu\text{g}/\text{L}$, whereas in air it is $1 \mu\text{g}/\text{m}^3$.

Upper bound cancer risk assessment: A qualifying statement indicating that the cancer risk estimate is not a true value in that the dose-response modeling used provides a value that is not likely to be an underestimate of the true value. The true value may be lower than the upper bound cancer risk estimate, and it may even be close to zero. This results from the use of a statistical upper confidence limit and from the use of conservative assumptions in deriving the cancer risk estimate.

Upper 95% confidence limit: Assuming random and normal distribution, this is the range of values below which a value will fall 95% of the time.

Voluntary risk: Risk that an individual has consciously decided to accept.

SAFETY PHARMACOLOGY

Action potential amplitude (APA): The amplitude of the cardiac action potential in millivolts (mV) and is a measure of the total amplitude of the action potential from initial resting level to peak depolarization. APA is determined by the combined activity of cardiac channels and transient potassium channels.

Action potential duration (APD): The duration of the cardiac action potential in milliseconds, measured from the initial upstroke to the point of return to either 60% (APD₆₀) or 90% (APD₉₀) of the initial resting potential. APD prolongation corresponds to an increase in the electrocardiographic QT interval.

Basic cycle length (BCL): In cardiac electrophysiology, is the BCL of repetitive stimulation and is the inverse of stimulation frequency. BCL = 2, 1, and 0.5 s, respectively, corresponds to bradycardic (30 beats/min), normocardic (60 beats/min), and tachycardic (120 beats/min) heart rates.

hERG: Refers to both the gene that encodes the pore-forming subunit of human cardiac ether-a-go-go-related potassium channel and the protein subunit itself. hERG channels are responsible for the delayed rectifier potassium current (I_{Kr}) that regulates action potential repolarization.

IC₅₀: Refers to the concentration or dose of test article that produces 50% inhibitory response in a test system assay.

Purkinje cells: Modified cardiac muscle cells specialized for the conduction of electric excitation from the atrio-ventricular node through the ventricular septum and throughout the walls of the ventricle. Purkinje cells are organized into fiber bundles that can be readily dissected from the working myocardium and studied in vitro using electrophysiological recording methods.

Purkinje fiber stimulation frequency: The repetition rate for the application of brief electric shocks to a Purkinje fiber preparation. A frequency rate of 1 Hz corresponds to 1 shock/s and represents the normal heart rate of 1 beat/s. Frequencies of 0.5 and 2 Hz correspond to bradycardic and tachycardic heart rates.

QT interval: The time interval in the electrocardiogram extending from the start of the QRS complex to the end of the T wave and is the measure of the duration of ventricular depolarization and repolarization. The QRS complex corresponds to the upstroke of the cardiac action potential, and the end of the T wave corresponds to the return of the action potential baseline.

Resting membrane potential (RMP): In cardiac electrophysiology, RMP is the resting membrane potential in mV and is obtained from the membrane voltage measured immediately before the action potential upstroke. RMP is controlled primarily by inwardly rectifying potassium channels. A decrease in RMP may indicate inhibition of the inward rectifier current.

V_{max}: In cardiac electrophysiology, this is the maximum rate of depolarization measured in Volts/seconds (V/s) obtained by taking the first derivative of the rising phase of the action potential. V_{max} amplitude is determined primarily by the activity of cardiac sodium channels. A decrease in V_{max} indicates sodium channel blockade and corresponds to a broadening of the electrographic QRS interval and, potentially, a slowing of conduction velocity in the intact heart.

REFERENCES

- Balls, M., Blaauboer, B., Brusick, D., Frazier, J., Lamb, D., Pemberton, M., Reinhart, C., Roberfroid, M., Rosenkrantz, H., Schmid, B., Spielmann, H., Stamatii, A.-L., and Walum, E. (1990) *Report and Recommendations of the CAA/ERGATT Workshop on the Validation of Toxicity Test Procedures*, ALTA 18,313.
- Cronin, E. (1980) *Contact Dermatitis*, Churchill Livingstone, New York, Chapters 1–17.
- Environ Corporation (1980) *Risk Assessment Guidance Manual*, AlliedSignal, Inc., Morristown, NJ.
- Hallenbeck, W.G. and Cunningham, K.M., eds. (1986) *Quantitative Risk Assessment for Environmental and Occupational Health*, Lewis Publishers, Chelsea, MI, Appendix 2.
- Klaassen, C.D., Amdur, M.O., and Doull, J., eds. (1991) *Casarett and Doull's Toxicology, The Basic Science of Poisons*, 4th edn., Pergamon Press, New York.
- Maronpot, R.R. (1991) *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, pp. 127–129.
- Marzulli, F.N. and Maibach, H.I., eds. (1977) *Dermatotoxicology*, 2nd edn., Hemisphere Publishing Corporation, Washington, DC.
- Middle Atlantic Reproduction and Teratology Association (1989) *A Compilation of Terms Used in Developmental Toxicity Evaluations*.
- Morris, W., ed. (1978) *The American Heritage Dictionary of the English Language*, New College edition, Houghton Mifflin Company, Boston, MA.
- Rand, G.M., ed. (1995) *Fundamentals of Aquatic Toxicology*, 2nd edn., Taylor & Francis Group, Washington, DC.
- Stedman's Medical Dictionary, 25th edn., Williams & Wilkins, Baltimore, MD, 1990.
- United States Environmental Protection Agency (1984) *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Hazard Evaluation Division, Guidance for Evaluation of Dermal Sensitization.
- United States Environmental Protection Agency (1989) *Glossary of Terms Related to Health, Exposure and Risk Assessment*, Air Risk Information Support Center, EPA No.450/3-88/016.
- United States Food and Drug Administration (2007) www.fda.gov

Glossary: Alphabetical

Abbreviated new drug application (ANDA): An ANDA contains data that, when submitted to FDA's Center for Drug Evaluation and Research, Office of Generic Drugs, provides for the review and ultimate approval of a generic drug product. Generic drug applications are called "abbreviated" because they are generally not required to include preclinical (animal) and clinical (human) data to establish safety and effectiveness. Instead, a generic applicant must scientifically demonstrate that its product is bioequivalent (i.e., performs in the same manner as the innovator drug). Once approved, an applicant may manufacture and market the generic drug product to provide a safe, effective, low-cost alternative.

Aberration: In developmental toxicology, a minor structural change. It may be a retardation (a provisional delay in morphogenesis), a variation (external appearance controlled by genetic and extragenetic factors), or a deviation (resulting from altered differentiation).

Ablepharia: Absence or reduction of the eyelid(s).

Abrachius: Without arms, forelimbs.

Absorbed dose: The amount of a substance penetrating across the exchange boundaries of an organism and into body fluids and tissues after exposure.

Acanthosis: In dermatology, hypertrophy of the stratum spinosum and granulosum.

Acardia: Absence of the heart.

Acaudia (anury): Agenesis of the tail.

Acceptable daily intake (ADI): A value used for noncarcinogenic effects that represents a daily dose that is very likely to be safe over an extended period of time. An ADI is similar to a reference dose (RfD) but less strictly defined.

Accessory spleen: An additional spleen.

Acephaly: Congenital absence of the head.

Achondroplasia: A hereditary defect in the formation of epiphyseal cartilage, resulting in a form of dwarfism with short limbs, normal trunk, small face, normal vault, etc.

Acrania: Partial or complete absence of the skull.

Action potential amplitude (APA): The amplitude of the cardiac action potential in mV and is a measure of the total amplitude of the action potential from initial resting level to peak depolarization. APA is determined by the combined activity of cardiac channels and transient potassium channels.

Action potential duration (APD): The duration of the cardiac action potential in milliseconds, measured from the initial upstroke to the point of return to either 60% (APD₆₀) or 90% (APD₉₀) of the initial resting potential. APD prolongation corresponds to an increase in the electrocardiographic QT interval.

Activated partial thromboplastin time: A measure of the relative activity of factors in the intrinsic clotting sequence and the common pathway necessary in normal blood coagulation.

Active ingredient: Any component that provides pharmacological activity or other direct effects in the diagnosis, cure, mitigation, treatment, or prevention of disease or affects the structure or any function of the body of man or animals.

Acystia: Absence of the urinary bladder.

Adactyly: Absence of digits.

Adduct: The covalent linkage or addition product between an alkylating agent and cellular macromolecules such as protein, RNA, and DNA.

Adjuvant (immunological): A material that enhances an immune response but does not confer immunity by itself. Examples include oil emulsions, aluminum salts, and toll-like receptor agonists. Complete Freund's adjuvant (CFA) was first developed by Jules Freund in the 1940s and contains a water-in-oil emulsion and mycobacterial cell fragments. Incomplete Freund's Adjuvant does not contain the mycobacterial cell fragments.

ADME: Absorption, distribution, metabolism, and excretion.

Administered dose: The amount of a substance given to a human or test animal in determining dose-response relationships, especially through ingestion or inhalation (see Applied dose). Administered dose is actually a measure of exposure, because even though the substance is "inside" the organism, once ingested or inhaled, administered dose does not account for absorption (see Absorbed dose).

Agastria: Absence of the stomach.

Agenesis: Absence of an organ or part of an organ.

Agenesis of the kidney: Absence of the kidney(s).

Agenesis of the lung (lobe): Complete absence of a lobe of the lung.

Aggregate risk: The sum of individual increased risks of an adverse health effect in an exposed population.

Aglossia: Absence of the tongue.

Agnathia: Absence of lower jaw (mandible).

Akinesia: Absence or the loss of power of voluntary motion; immobility.

Alanine amino transferase (ALT): An enzyme, primarily of liver origin, whose blood levels can rise in response to hepatocellular toxicity. Also known as serum glutamic pyruvic transaminase (SGPT).

Albumin: The most abundant blood protein synthesized by the liver.

Alkaline phosphatase: An enzyme whose blood levels can rise in response to hepatobiliary disease or

increased osteoblastic (bone cell) activity. Serum alkaline phosphatase activity can decrease in fasted rats because the intestinal isozyme is an important component of serum enzyme activity.

Alkylating agent: A chemical compound that has positively charged (electron-deficient) groups that can form covalent linkages with negatively charged portions of biological molecules such as DNA. The covalent linkage is referred to as an adduct and may have mutagenic or carcinogenic effects on the organism. The alkyl species is the radical that results when an aliphatic hydrocarbon loses one hydrogen atom to become electron deficient. Alkylating agents react primarily with guanine, adding their alkyl group to N7 of the purine ring.

Allogeneic: From a different genetic background. In the context of immunotoxicology, this usually refers to the use of genetically dissimilar cells in *in vitro* assays to elicit a cell-mediated immune reaction.

Altered focus: A histologically identifiable clone of cells within an organ that differs phenotypically from the normal parenchyma. Foci of altered cells usually result from increased cellular proliferation, represent clonal expansions of initiated cells, and are frequently observed in multistage animal models of carcinogenesis. Foci of cellular alteration are most commonly observed in the liver of carcinogen-treated rodents and are believed by some to represent preneoplastic lesions.

Anal atresia: Congenital absence of the anus.

Anemia: Any conditions in which RBC count, hemoglobin concentration, and hematocrit are reduced.

Anencephaly: Congenital absence of the cranial vault with missing or small brain mass.

Aneuploidy: An abnormal number of chromosomes in a cell or organisms that is not an exact multiple of the haploid number.

Anisocytosis: Variations in the size of red blood cells.

Anomaly or abnormality: In developmental toxicology, a morphological or functional deviation from normal limit. It can be a malformation or a variation.

Anophthalmia: Absence of eye(s).

Anorchism: Congenital absence of one or both testes.

Anotia: Absence of the external ear(s).

Anterior chamber: The aqueous-containing cavity of the eye, bounded by the cornea anteriorly, the chamber angle structures peripherally, and the iris and lens posteriorly.

Antibody: Are also referred to as immunoglobulins (Ig). They consist of two basic units: the antigen-binding fragment (Fab), which contains variable regions coding for antigen recognition, and the constant fragment (Fc), which determines the function of the antibody. Based on the Fc region, immunoglobulins are designated IgA, IgD, IgE, IgG, or IgM. The cross-linking of antibody molecules on the surface of a cell leads to the activation of complement, resulting in the destruction of the target by lytic cells or in phagocytosis by macrophages.

Antibody dependant cell-mediated cytotoxicity (ADCC): Cell-mediated immunity (CMI) in which a specific antibody binds to a target cell, targeting it for cytolytic activity by an effector cell (generally a macrophage or NK cell).

Antibody forming cell (AFC)/Plaque forming cell (PFC) assay: The AFC assay measures the ability of animals to produce either IgM or IgG antibodies against a T-dependent or T-independent antigen following *in vivo* sensitization. Due to the involvement of multiple cell populations in mounting an antibody response, the AFC assay actually evaluates several immune parameters simultaneously. It is considered to be one of the most sensitive indicator systems for immunotoxicology studies.

Antigen: A molecule that is the subject of a specific immune reaction. Antigens are recognized in a cognate fashion by either immunoglobulins or the antigen receptor on the surface of T cells. Antigens are usually proteinaceous in nature.

Antibody presenting cell (APC): Cells that are responsible for making antigens accessible to immune effector and regulatory cells. Following internalization and degradation of the antigen (e.g., by phagocytes), a fragment of the antigen molecule is presented on the APC cell surface in association with histocompatibility molecules. The resulting complex is subsequently recognized either by B cells via surface-bound Ig molecules or by T cells via the T cell antigen receptor (TCR). The induction of a specific immune response then proceeds. Representative APC includes macrophages, dendritic cells, and certain B cells.

Aphalangia: Absence of a finger or a toe; corresponding metacarpals not affected.

Aplasia: Lack of development of an organ, frequently used to designate complete suppression or failure of development of a structure from the embryonic primordium.

Aplasia of the lung: The trachea shows rudimentary bronchi, but pulmonary and vascular structures are absent.

Apodia: Absence of one or both feet.

Applied dose: The amount of a substance given to a human or test animal in determining dose-response relationships, especially through dermal contact. Applied dose is actually a measure of exposure, since it does not take absorption into account (see Absorbed dose).

Aproctia: Imperforation or absence of anus.

Arrhinia: Absence of nose.

Arthrogryposis: Persistent flexure or contracture of a joint; flexed paw (bent at wrist) is the most common form of arthrogryposis.

Aspartate aminotransferase (AST): An enzyme whose blood levels can rise in response to hepatotoxicity, muscle damage, or hemolysis. Also known as serum glutamic oxaloacetic transaminase (SGOT).

Aspermia: No ejaculate.

Asthenozoospermia: More than 50% spermatozoa with poor (<2 grade) forward progression.

Astomia: Absence of oral orifice.

Ataxia: Incoordination; the inability to coordinate the muscles in the execution of voluntary movement.

Autoimmunity: Reaction of the immune system against the host organism. In the context of drug development, autoimmunity may take the form of escape from tolerance, as when a drug modifies a host antigen, which is subsequently seen as foreign. Drug-induced autoimmunity may also result from bystander damage to host tissues from a drug-specific immune reaction.

Azoospermia: No spermatozoa in the ejaculate.

Azotemia: An increase in serum urea nitrogen and/or creatinine levels.

Base substitution: The substitution of one or more base(s) for another in the nucleotide sequence.

Basic cycle length (BCL): In cardiac electrophysiology is the BCL of repetitive stimulation and is the inverse of stimulation frequency. BCL = 2, 1, and 0.5 s, respectively, corresponds to bradycardic (30 beats/min), normocardic (60 beats/min), and tachycardic (120 beats/min) heart rates.

B cell/B lymphocyte: Lymphocytes that recognize antigen via surface-bound Ig. B cells that have been exposed to cognate antigen subsequently proliferate and differentiate into plasma cells, which are responsible for producing specific antibody. B cells differentiate in the bone marrow in mammals and in an organ known as the bursa of Fabricius in birds.

Benign: A classification of anticipated biological behavior of neoplasms in which the prognosis for survival is good. Benign neoplasms grow slowly, remain localized, and usually cause little harm to the patient.

Bioaccumulation: Ecotoxicology term describing a process by which chemicals are taken up by aquatic organisms directly from water as well as from exposure through other routes, such as consumption of food and sediment containing chemicals.

Bioaccumulation factor (BAF): The ratio of tissue chemical residue to chemical concentration in an external environmental phase (i.e., water, sediment, or food). BAF is measured as steady state in situations where organisms are exposed from multiple sources (i.e., water, sediment, and food), unless noted otherwise.

Biochemical oxygen demand (BOD): Sometimes called *biological oxygen demand*, a measure of the rate at which molecular oxygen is consumed by microorganisms during oxidation of organic matter. The standard test is the 5-day BOD test, in which the amount of dissolved oxygen required for oxidation over a 5-day period is measured. The results are measured in mg of oxygen/L (mg/L) or parts per million (ppm).

Bioconcentration: A process by which there is a net accumulation of a chemical directly from water into aquatic

organisms resulting from simultaneous uptake (e.g., by gill or epithelial tissue) and elimination.

Bioconcentration factor (BCF): A term describing the degree to which a chemical can be concentrated in the tissues of an organism in the aquatic environment as a result of exposure to waterborne chemical. At steady state during the uptake phase of a bioconcentration test, the BCF is a value that is equal to the concentration of a chemical in one or more tissues of the exposed aquatic organisms divided by the average exposure water concentration of the chemical in the test.

Biodegradation: The transformation of a material resulting from the complex enzymatic action of microorganisms (e.g., bacteria, fungi). It usually leads to disappearance of the parent chemical structure and to the formation of smaller chemical species, some of which are used for cell anabolism. Although typically used with reference to microbial activity, it may also refer to general metabolic breakdown of a substance by any living organism.

Biologic license application (BLA): Biological products are approved for marketing under the provisions of the Public Health Service Act (PHS Act). The Act requires a firm who manufactures a biologic for sale in interstate commerce to hold a license for the product. A biologics license application is a submission that contains specific information on the manufacturing processes, chemistry, pharmacology, clinical pharmacology, and the medical effects of the biological product. If the information provided meets FDA requirements, the application is approved, and a license is issued allowing the firm to market the product.

Biological product: A biological product is any virus, serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries. Biological products are a subset of "drug products" distinguished by their manufacturing processes (biological process vs. chemical process). In general, the term "drugs" includes biological products.

Biological significant effect: A response in an organism or other biological system that is considered to have a substantial or noteworthy effect (positive or negative) on the well-being of the biological system. Used to distinguish from statistically significant effects or changes, which may or may not be meaningful to the general state of health of the system.

Biomaterial: A material that has direct or indirect patient contact. A biomaterial (also termed a *biomedical material*) may be composed of any synthetic or natural rubber or fiber, polymeric or elastomeric formulation, alloy, ceramic, bonding agent, ink, or other nonviable substance, including tissue rendered nonviable, used as a device or any part thereof.

Blanching: In dermal toxicology, to take color from, to bleach. Characterized by a white or pale discoloration of the chemically exposed area of the skin due to decreased blood (ischemia).

Blepharitis: Inflammation of the eyelids.

Blepharospasm: Involuntary spasm of the lids.

Brachydactyly: Shortened digits.

Brachyury (short tail): Tail that is reduced in length.

Bulbous rib: Having a bulge or balloon-like enlargement somewhere along its length.

Cancer potency factor (CPF): The statistical 95% upper confidence limit on the slope of the dose–response relationship at low doses for a carcinogen. Values are in units of lifetime risk per unit dose (mg/kg/day). A plausible upper bound risk is derived by multiplying the extended lifetime average daily dose (LADD) by the CPF.

Cardiomegaly: Hypertrophy (enlargement) of the heart.

Cardiovascular situs inversus: Mirror-image transposition of the heart and vessels to the other side of the body.

Case-control study: A retrospective epidemiologic study in which individuals with the disease under study (cases) are compared with individuals without the disease (controls) in order to contrast the extent of exposure in the diseased group with the extent of exposure in the controls.

Catalepsy: Condition in which there is waxy rigidity of the limbs that may be placed in various positions that will be maintained for a time.

Cataract: An opacity of the lens or its capsule.

CBER: FDA's Center for Biologics Evaluation and Research—the division charged with regulating biological products.

Cluster of differentiation (CD): The CD series in immunology is used to denote cell surface markers (e.g., CD4, CD8). These markers, used experimentally as a means of identifying cell types, also serve physiological roles usually as receptors or ligands. The CD nomenclature was standardized in 1982 and has been expanded since then as new molecules are characterized. The CD nomenclature is especially useful in immunophenotyping immune cell types based on the proteins present on their surface.

CDER: FDA's Center for Drug Evaluation and Research—the division charged with developing and enforcing policy with regard to the safety, effectiveness, and labeling of all drug products for human use.

Ceiling limit: A concentration limit in the work place that should not be exceeded, even for a short time, to protect workers against frank health effects.

Cell-mediated immunity (CMI): Antigen-specific reactivity mediated primarily by T cells. CMI may take the form of immunoregulatory activity (mediated by CD4+ helper T cells) or immune effector activity (mediated by CD8+ killer T cells). Other forms of direct cellular activity in host defense (e.g., NK cells, macrophages) are not antigen specific and are more accurately referred to as innate immunity.

Cephalocele: A protrusion of a part of the cranial contents, not necessarily neural tissue.

CFR: Code of Federal Regulations (United States).

Chemical oxygen demand (COD): COD is measured instead of BOD when organic materials are not easily degraded by microorganisms. Strong oxidizing agents (e.g., potassium permanganate) are used to enhance oxidation. COD values will be larger than BOD values.

Chemokine/chemotactic cytokines: Small chemoattractant proteins important in the stimulation and migration of immune cells. Chemokines are divided into four groups depending on the positions and spacing of the cysteine residues.

Chemosis: In ophthalmology, intense edema of the conjunctiva. The conjunctiva is loose fibrovascular connective tissue that is relatively rich in lymphatics and responds to noxious stimuli by swelling to the point of prolapse between the lids.

Choristoma: A mass of well-differentiated cells from one organ included within another organ, for example, adrenal tissue present in the lung.

Choroid: The vascular middle coat between the retina and sclera of the eye.

Chromosomal aberration: A numerical or structural chromosomal abnormality.

Ciliary body: Portion of the uveal tract between the iris and the choroid of the eye consisting of ciliary processes and the ciliary muscle.

Class testing: The testing of medical device plastics for biological reactivity according to predetermined testing requirements defined by the United States Pharmacopeia (USP).

Clastogen: An agent that produces structural changes of chromosomes.

Clonic convulsion: A convulsion in which the muscles alternately relax and contract.

Clonus: A form of movement characterized by contractions and relaxations of a muscle.

Cocarcinogen: An agent not carcinogenic alone but that potentiates the effect of a known carcinogen.

Cocarcinogenesis: The augmentation of neoplasm formation by simultaneous administration of a genotoxic carcinogen and an additional agent (cocarcinogen) that has no inherent carcinogenic activity by itself.

Cohort study: A study of a group of persons sharing a common experience (e.g., exposure to a substance) within a defined time period; the experiment is used to determine if an increased risk of a health effect (disease) is associated with that exposure.

Combination (medical device) product: A product containing both a drug and a device component that are physically, chemically, or otherwise combined to result in a medical product that is used therapeutically as a single entity. The medical device component must be evaluated for safety according to device requirements, the drug component must be evaluated for safety as per drug requirements, and the safety of the finished combined product must be also be evaluated.

- Complement:** A group of approximately 20 protein precursor molecules assemble into a complex that intercalates into the membrane of a cell and forms a pore resulting in osmotic lysis of the target cell.
- Conceptus:** The sum of derivatives of a fertilized ovum at any stage of development from fertilization until birth. Also, term used when the stage of prenatal development at the time of initial insult is not known; also referred to as *embryo–fetus*.
- Confidence limit:** The confidence interval is a range of values that has a specified probability (e.g., 95%) of containing a given parameter or characteristic. The confidence limit often refers to the upper value of the range (e.g., upper confidence limit).
- Conjunctiva:** Mucous membrane that lines the posterior aspect of the eyelids (palpebral conjunctiva) and the anterior sclera (bulbar conjunctiva).
- Conjunctivitis:** Inflammation of the conjunctiva.
- Contact dermatitis:** A delayed type of induced sensitivity (allergy) of the skin with varying degrees of erythema, edema, and vesiculation, resulting from cutaneous contact with a specific allergen.
- Contact urticaria:** Wheal-and-flare response elicited with 30–60 min after cutaneous exposure to test substance. May be IgE mediated or nonimmunologically mediated.
- Convulsion:** A violent spasm of the face, trunk, or extremities.
- Cornea:** Transparent portion of the outer coat of the eyeball forming the anterior wall of the anterior chamber.
- Corpus luteum:** The yellow endocrine body formed in the ovary at the site of the ruptured Graafian follicle.
- Corrosion:** In dermal toxicology, direct chemical action on normal living skin that results in its disintegration and irreversible alteration at the site of contact. Corrosion is manifested by ulceration and necrosis with subsequent scar formation.
- Craniorachischisis:** Exencephaly and holorrhachischisis (fissure of the spinal cord).
- Cranioschisis:** Abnormal fissure of the cranium; may be associated with meningocele or encephalocele.
- Creatine kinase (CK):** An enzyme that is concentrated in skeletal muscle, brain, and heart tissue.
- Creatinine:** The end product of creatine metabolism in muscle. Elevated blood levels can indicate renal (glomerular) injury.
- Critical endpoint:** A chemical may elicit more than one toxic effect (endpoint), even in one test animal, in tests of the same or different duration (acute, subchronic, and chronic exposure studies). The doses that cause these effects may differ. The critical endpoint used in the dose–response assessment is the one that occurs at the lowest dose. In the event that data from multiple species are available, it is often the most sensitive species that determines the critical endpoint. This term is applied in the derivation of risk reference doses (RfDs).
- Cross-sectional study:** An epidemiologic study assessing the prevalence of a disease in a population. These studies are most useful for conditions or diseases that are not expected to have a long latent period and do not cause death or withdrawal from the study population. Potential bias in case ascertainment and exposure duration must be addressed when considering cross-sectional studies.
- Cryptorchidism (undescended testes, ectopic testes):** Failure of the testes to descend into the scrotum (can be unilateral).
- Cumulative irritation:** In dermal toxicology, primary irritation resulting from repeated exposures to materials that do not in themselves cause acute primary irritation.
- Cyclopia:** In developmental toxicology, one central orbital fossa with none, one, or two globes.
- Cytokine:** Small peptides produced primarily by cells of the immune system, particularly helper T cells. Cytokines are grouped into nonexclusive categories including interleukins, tumor necrosis factors, interferons, colony-stimulating factors, and various miscellaneous cytokines. Related molecules include peptide growth factors, transforming growth factors, and chemokines. Cytokines form an interactive network with both hormones and neuropeptides. Cytokines may be referred to in the older literature as lymphokines.
- Cytotoxic T-lymphocyte (CTL):** A subset of CD8+ T cells able to kill target cells following the induction of a specific immune response. The mechanism of lysis appears to be a combination of direct lysis by extravasation of lytic molecules (such as perforins and granzymes), as well as the induction of apoptosis in the target cell. The measurement of CTL activity is a sensitive indicator of cell-mediated immunity (CMI).
- De minimus risk:** From the legal maxim, “de minimus non curat lex” or the law is not concerned with trifles. As relates to risk assessment of carcinogens, it is commonly interpreted to mean that a lifetime risk of 1×10^{-6} is a *de minimus* level of cancer risk (i.e., insignificant and therefore acceptable) and is of no public health consequence.
- Deflection:** In developmental toxicology, a turning, or state of being turned, aside.
- Deformity:** Distortion of any part or general disfigurement of the body.
- Delayed-type hypersensitivity (DTH):** A form of cell-mediated immunity (CMI) in which secondary exposure to an antigen results in an inflammatory reaction mediated by CD4+ T cells.
- Dermatitis:** Inflammation of the skin.
- Desquamation:** The shedding of the cuticle in scales or the outer layer of any surface. In dermal toxicology, to shred, peel, or scale off, as the casting off of the epidermis in scales or shred or the shedding of the outer layer of any surface.
- Deviation:** Variation from the regular standard or course.

- Dextrogastria:** Having the stomach on the right side of the body.
- Dextrocardia:** Location of the heart in the right side of the thorax; a developmental disorder that is associated with total or partial situs inversus (transposition of the great vessels and other thoracoabdominal organs) or occurs as an isolated anomaly.
- Direct carcinogen:** Carcinogens that have the necessary structure to directly interact with cellular constituents and cause neoplasia. Direct acting carcinogens do not require metabolic conversion by the host to be active. They are considered genotoxic because they typically undergo covalent binding to DNA.
- Direct contact:** When the materials of a medical device are in direct (i.e., intimate) contact with the surface or tissues of the body (e.g., adhesive bandages, pacemaker leads).
- Dispersion model:** A mathematical model or computer simulation used to predict the movement of airborne or waterborne contaminants. Models take into account a variety of mixing mechanisms that dilute effluents and transport them away from the point of emission.
- Displaced rib:** Out of normal position.
- Dose:** The amount of substance administered to an animal or human generally expressed as the weight or volume of the substance per unit of body weight (e.g., mg/kg, mL/kg).
- Dose–response relationship:** A relationship between the dose, often actually based on “administered dose” (i.e., exposure) rather than absorbed dose, and the extent of toxic injury produced by that chemical. Response can be expressed as either the severity of injury or proportion of exposed subjects affected. A dose–response assessment is one of the steps in a risk assessment.
- Drug:** A substance recognized by an official pharmacopoeia or formulary. A substance intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease. A substance (other than food) intended to affect the structure or any function of the body. A substance intended for use as a component of a medicine but not a device or a component, part, or accessory of a device. Biological products are included within this definition and are generally covered by the same laws and regulations, but differences exist regarding their manufacturing processes (chemical process vs. biological process.)
- Drug label:** The FDA-approved label is the official description of a drug product that includes indication (what the drug is used for); who should take it; adverse events (side effects); instructions for uses in pregnancy, children, and other populations; and safety information for the patient. Labels are often found inside drug product packaging.
- Duration of exposure:** Generally referred to in toxicology as acute (one time), subacute (repeated over several weeks), subchronic (repeated for a fraction of a lifetime), and chronic (repeated for nearly a lifetime).
- Dust:** The airborne state of a chemical that is solid at room temperature but is dispensed into a particulate atmosphere.
- Dysarthria:** In neurotoxicology, disturbance of articulation due to emotional stress or to paralysis, incoordination, or spasticity of muscles used in vocalizing.
- Dysgenesis:** Defective development; malformation.
- Dyskinesia:** Difficulty in performing voluntary movements; a movement disorder characterized by insuppressible, stereotyped, and automatic movements.
- Dysmelia:** Absence of a portion of one or several limbs.
- Dysplasia:** Disordered tissue formation characterized by changes in size shape and orientational relationships of adult types of cells. Primarily seen in epithelial cells.
- Dystocia:** Abnormal labor.
- Dystonia:** Abnormal tonicity (hyper or hypo) in any tissues.
- EC₅₀ (median effective concentration):** The concentration of chemical in water to which test organisms are exposed that is estimated to be effective in producing some sublethal response in 50% of the test organisms. The EC₅₀ is usually expressed as a time-dependent value (e.g., 24 h or 96 h EC₅₀). The sublethal response elicited from the test organisms as a result of exposure to the chemical must be clearly defined (e.g., test organisms may be immobilized, lose equilibrium, or undergo physiological or behavioral changes).
- Ectocardia:** Displacement of the heart inside or outside the thorax.
- Ectopic:** Out of the normal place.
- Ectopic esophagus:** Displacement of the esophagus (description of position should be included).
- Ectopic pinna:** Displaced external ear.
- Ectrodactyly:** Absence of all or of only a part of digit (partial ectrodactyly).
- Ectromelia:** Aplasia or hypoplasia of one or more bones of one or more limbs (this term includes amelia, hemimelia, and phocomelia).
- Eczema:** Inflammatory condition in which the skin becomes red and small vesicles, crusts, and scales develop.
- Edema:** An excessive accumulation of serous fluid or water in cells, tissues, or serous cavities.
- Enzyme-linked immunosorbent assay (ELISA):** A type of immunoassay in which specific antibodies are used to both capture and detect antigens of interest. The most common type is the “sandwich” ELISA in which antibodies are bound to a substrate such as a plastic culture plate, and a second labeled antibody is used to detect the bound molecules.
- Encephalocele:** A partial protrusion of brain through an abnormal cranial opening; not as severe as exencephaly.
- Endemic:** Present in a community or among a group of people; said of a disease prevailing continually in a region.

- Environmental fate:** The destiny of a chemical or biological pollutant after release into the environment. Environmental fate involves temporal and spatial considerations of transport, transfer, storage, and transformation.
- Epigenetic:** Change in phenotype without a change in DNA structure. One of the two main mechanisms of carcinogen action, epigenetic carcinogens are nongenotoxic, that is, they do not form reactive intermediates that interact with genetic material in the process of producing or enhancing neoplasm formation.
- Erythema:** An inflammatory redness of the skin, as caused by chemical poisoning or sunburn, usually a result of congestion of the capillaries.
- Eschar:** A dry scab, thick coagulated crust or slough formed on the skin as a result of a thermal burn or by the action of a corrosive or caustic substance.
- Estrus:** Phase of the sexual cycle of female mammals characterized by willingness to mate.
- Exencephaly:** Brain outside of the skull as a result of a large cranial defect.
- Exfoliation:** To remove in flakes or scales, peel. To cast off in scales, flakes, or the like. To come off or separate, as scales, flakes, sheets, or layers. In dermal toxicology, detachment and shedding of superficial cells of an epithelium or from any tissue surface. Scaling or desquamation of the horny layer of epidermis, which varies in amount from minute quantities to shedding the entire integument.
- Exomphalos:** Congenital herniation of abdominal viscera into umbilical cord.
- Exophthalmos:** Abnormal protrusion of the eyeball.
- Exophthalmos:** Protrusion of the eyeball ("pop" eye).
- Exposure:** Contact of an organism with a chemical, physical, or biological agent. Exposure is quantified as the amount of the agent available at the exchange boundaries of the organism (e.g., skin, lungs, digestive tract) and available for absorption.
- Exposure assessment:** The determination of the extent of human exposure.
- Exposure frequency:** The number of times an exposure occurs in a given period. The exposure(s) may be continuous, discontinuous but regular (e.g., once daily), or intermittent.
- Extract:** A solution produced by the incubation of a material/medical device in an appropriate vehicle. After incubation, the vehicle contains the soluble chemicals (or leachables) that have dissolved out of, or off, the material/medical device.
- Extrapolation:** An estimate of response or quantity at a point outside the range of the experimental data. Also refers to the estimation of a measured response in a different species or by a different route than that used in the experimental study of interest (i.e., species to species, route to route, acute to chronic, high to low).
- Fasciculations:** Involuntary contractions, or twitching, of groups of muscle fibers.
- Fate:** Disposition of a material in various environmental compartments (e.g., soil or sediment, water, air, biota) as a result of transport, transformation, and degradation.
- Fecundity:** Ability to produce offspring rapidly and in large numbers.
- Fence line concentration:** Modeled or measured concentrations of pollutants found at the boundaries of a property on which a pollution source is located. Usually assumed to be the nearest location at which an exposure of the general population could occur.
- Feticide:** The destruction of the fetus in the uterus.
- Fibrinogen:** A glycoprotein that is involved in the formation of fibrin.
- Fluorescein (fluorescein sodium):** A fluorescent dye, the simplest of the fluorane dyes and the mother substance of eosin, which is commonly used intravenously in ophthalmology to determine the state of adequacy of circulation in the retina and a lesser degree the choroid and iris. Another important use is to detect epithelial lesions of the cornea and conjunctiva. Peak excitation occurs with light at a wavelength between 485 and 500 m μ m, and peak emission occurs between 520 and 530 m μ m.
- Fovea:** Depression in the macula of the eye adapted for most acute vision.
- Frameshift mutation:** A mutation in the genetic code in which one base or two adjacent bases are inserted or deleted to the nucleotide sequence of a gene.
- Frank effect level (FEL):** Related to biological responses to chemical exposures (compare with NOAEL and LOEL); the exposure level that produces an unmistakable adverse health effect (such as inflammation, severe convulsions, or death).
- Fume:** The airborne state of a chemical that is liquid or solid at room temperature and pressure but is heated and allowed to condense into a particulate atmosphere.
- Fundus:** The posterior portion of the eye visible through an ophthalmoscope.
- Gamete:** A male (spermatozoa) or female (ovum) reproductive cell.
- Gamma glutamyltransferase (γ GT):** An enzyme of liver origin, whose blood concentration can be elevated in hepatobiliary disease.
- Gas:** The airborne state of a chemical that boils at or below room temperature and pressure.
- Gastroschisis:** Fissure of the abdominal wall (median line) not involving the umbilicus, usually accompanied by protrusion of the small part of the large intestine, not covered by membranous sac.
- Gene mutation:** A detectable permanent change (point mutation, insertion, or deletion) within a single gene or its regulating sequences.
- Genotoxic carcinogen:** An agent that interacts with cellular DNA either directly in its parent form (direct carcinogen) or after metabolic biotransformation.

- Geometric standard deviation (GSD):** In inhalation toxicology, the relative dispersion of the MMAD such that a value approaching 1 indicates a monodisperse atmosphere.
- Globulin:** A group of blood proteins synthesized by lymphatic tissue in the liver.
- Gut-associated lymphoid tissue (GALT):** Lymphoid cells and tissues lining the mucosa that serve as the first point of contact with antigen encountered via this route. GALT comprises Peyer's patches, the appendix, tonsils, and mesenteric lymph nodes.
- Hapten:** Low molecular weight molecules that are not antigenic by themselves but that are recognized as antigens when bound to larger molecules, usually proteins.
- Hazard:** The inherent ability of a substance to cause an adverse effect under defined conditions of exposure.
- Hazard identification:** The determination of whether a particular chemical is or is not causally linked to particular health effects.
- Hazard index:** The ratio of the maximum daily dose (MDD) to the acceptable daily intake (ADI) used to evaluate the risk of noncarcinogens. A value of less than 1 indicates that the risk from the exposure is likely insignificant; a value greater than 1 indicates a potentially significant risk.
- Head-only exposure:** A system for exposing test animals via inhalation in which they are restrained in a tube in such a way that only their heads are exposed directly to the test material.
- Hemivertebra:** Presence of only one-half of a vertebral body.
- Hemolysis:** The destruction of red blood cells resulting in the liberation of hemoglobin into plasma.
- Hepatic lobe agenesis:** Absence of a lobe of the liver.
- Hepatomegaly:** Abnormal enlargement of the liver.
- hERG:** Refers to both the gene that encodes the pore-forming subunit of human cardiac ether-a-go-go-related potassium channel and the protein subunit itself. hERG channels are responsible for the delayed rectifier potassium current (I_{Kr}) that regulates action potential repolarization.
- Host defense:** The ability of an organism to protect itself against disease associated with exposure to infectious organisms, foreign tissue, or neoplasia. Host defense assays measure protection from infectious or neoplastic disease and are mediated by an immunological effect involving all components of the immune system—innate, cell-mediated, and humoral-mediated immunity (HMI). Host resistance assays are the only way to demonstrate immunological reserve.
- Human equivalent dose (HED):** A dose in humans anticipated to provide the same degree of effect as that observed in animals at a given dose. In drug development, the term HED is usually used to refer to the HED of the NOAEL.
- Humoral-mediated immunity (HMI):** Specific immune responses that are mediated primarily by humoral factors including antibodies and complement. The induction of HMI generally, although not exclusively, requires the cooperation of cellular immune mechanisms.
- Hybridoma:** A genetically engineered cell clone that produces antibodies of a single type (i.e., monoclonal antibodies). Monoclonal antibodies are highly specific for their cognate antigen and make highly useful tools for immunotoxicological studies.
- Hydrocephaly:** Enlargement of the head caused by abnormal accumulation of cerebrospinal fluid in subarachnoid cavity (external hydrocephaly) or ventricular system (internal hydrocephaly).
- Hydronephrosis:** Dilatation of the renal pelvis usually combined with destruction of renal parenchyma and often with dilation of the ureters (bilateral, unilateral). Note: This is a pathology term and should have histological confirmation.
- Hyperemia:** Excess of blood in a part due to local or general relaxation of the arterioles. Blood vessels become congested and give the area involved a reddish or red-blue color.
- Hyperkeratosis:** Hypertrophy and thickening of the stratum corneum.
- Hyperkinesia:** Excessive muscular activity.
- Hyperplasia:** A numerical increase in the number of phenotypically normal cells within a tissue or organ.
- Hypersensitivity:** A vigorous and often inappropriate immune response to seemingly innocuous antigens. Hypersensitivity is classified into subtypes depending on the mechanisms of action and the target cells or tissues.
- Hypertrophy:** Increase in the size of an organelle, cell, tissue, or organ within a living organism. To be distinguished from hyperplasia, hypertrophy refers to an increase in size rather than an increase in number. Excessive hyperplasia in a tissue may produce hypertrophy of the organ in which that tissue occurs.
- Hypoplasia of the lung:** Bronchial tree is poorly developed, and pulmonary tissue shows an abnormal histological picture (total or partial); incomplete development, smaller.
- Hypospadias:** Urethra opening on the underside of the penis or on the perineum (males) or into the vagina (females).
- IC₅₀:** Refers to the concentration or dose of test article that produces 50% inhibitory response in a test system assay.
- Icteric:** Relating to a jaundiced condition, typically as a result of elevated serum bilirubin levels.
- Identification threshold:** A limit (%) above which a drug impurity or degradation product should be identified.
- Immediately dangerous to life and health (IDLH):** A concentration representing the maximum level of a pollutant from which an individual could escape within 30 min without escape-impairing symptoms or irreversible health effects.

- Immunological reserve:** The concept that the immune response exhibits multiple immunological functions acting in an orchestrated manner, such that a decrease in one function is compensated by other immune functions. Immunological reserve prevents infectious or neoplastic disease due to acute reductions in one or two immune functions. This reserve would theoretically prevent a severe reduction in host resistance following a temporary immunosuppression of selected immune functions. This concept is important in the interpretation of immunotoxicology data.
- Immunostimulation:** Enhancement of immune function above an accepted baseline (control) level. Immunostimulation may be beneficial, for example, therapeutics designed to restore a suboptimal immune response. It may conversely be detrimental, as would be the case with autoimmunity or hypersensitivity.
- Immunosuppression:** Depression/reduction of immune function below an accepted baseline (control) level. Immunosuppression may result from inadvertent exposure to drugs or other chemical or physical agents, intentional modification for therapeutic reasons (e.g., organ transplantation), or following exposure to certain infectious agents (e.g., HIV). An important consideration in immunotoxicology is the ability to determine the amount of immunosuppression necessary to alter host defense. Immunosuppression may result in a state of immunodeficiency.
- Imperforate:** Not open; abnormally closed.
- In situ carcinoma:** A localized intraepithelial form of epithelial cell malignancy. The cells possess morphological criteria of malignancy but have not yet gone beyond the limiting basement membrane.
- Incidence:** The number of new cases of a disease within a specified time period. It is frequently presented as the number of new cases per 1,000, 10,000, or 100,000. The incidence rate is a direct estimate of the probability or risk of developing a disease during a specified time period.
- Incomplete ossification (delayed, retarded):** The extent of ossification is less than what would be expected for that developmental age, not necessarily associated with reduced fetal or pup weight.
- IND:** Investigational New Drug Application—a request to initiate a clinical study of a new drug product.
- Indirect contact:** When materials of a medical device do not contact the surface or tissues of the body, the materials of the device may influence the body. In this case, a solution or other materials that contact the device may become contaminated with leachables from the device that in turn contacts tissues of the body (e.g., intravenous infusion bag).
- Initiation:** The first step in carcinogenesis whereby limited exposure to a carcinogenic agent produces a latent but heritable alteration in a cell, permitting its subsequent proliferation and development into a neoplasm after exposure to a promoter.
- Initiator:** A chemical, physical, or biological agent that is capable of irreversibly altering the genetic component (DNA) of the cell. While initiators are generally considered to be carcinogens, they are typically used at low noncarcinogenic doses in two-stage initiation–promotion animal model systems. Frequently referred to as a “tumor initiator.”
- Injection:** In ophthalmology, congestion of blood vessels.
- Innate (natural or nonspecific) immunity:** Host defense mechanisms that do not require prior exposure to antigen; often are antigen nonspecific in nature. Nonspecific immunity is mediated by NK cells, macrophages, neutrophils, $\gamma\delta$ T cells, and complement.
- Intratracheal dosing:** A method of delivering, via a syringe and blunt needle, test material directly into the trachea of a test animal.
- Involuntary risk:** A risk that impinges on an individual without their awareness or consent.
- Iris:** The circular pigmented membrane behind the cornea and immediately in front of the lens; the most anterior portion of the vascular tunic of the eye. It is composed of the dilator and sphincter muscles and the two-layered posterior epithelium and mesodermal components that form the iris stroma.
- Iritis:** Inflammation of the iris, manifested by vascular congestion (hyperemia). An outpouring of serum proteins into the aqueous (flare) may accompany the inflammatory reaction.
- Irritant:** A substance that causes inflammation and other evidences of irritation, particularly of the skin, on first contact or exposure; a reaction of irritation not dependent on a mechanism of sensitization.
- Irritation:** A local reversible inflammatory response of normal living skin to direct injury caused by a single application of a toxic substance, without the involvement of an immunological mechanism.
- ISO:** International Standards Organization.
- Keratitis:** Inflammation of the cornea.
- Lactate dehydrogenase:** An enzyme found in several organs, including liver, kidney, heart, and skeletal muscle.
- Latency:** The period of time between exposure to an injurious agent and the manifestation of a response.
- LC₅₀ (median lethal concentration):** The concentration of a chemical required to cause death in 50% of the exposed population when exposed for a specified time period and observed for a specified period of time after exposure. Refers to inhalation exposure concentration in the context of air toxics and may refer to water concentration for tests of aquatic organisms. In aquatic toxicology, the LC₅₀ is often expressed as a time-dependent value (e.g., 24 h or 96 h LC₅₀).
- LC₁₀ (lethal concentration low):** The lowest concentration of a chemical required to cause death in some of the population after exposure for a specified period of time and observed for a specified period of time

after exposure. Refers to inhalation time exposure in the context of air toxics (may refer to water concentration for tests of aquatic organisms).

LD₅₀ (median lethal dose): The dose of a chemical required to cause death in 50% of the exposed population after noninhalation exposure (e.g., injection, ingestion) for a specified observation period after exposure.

LD_{Lo} (lethal dose low): The lowest dose of a chemical required to cause death in some of the population after noninhalation exposure (e.g., injection, ingestion) for a specified observation period after exposure.

Lens: A transparent biconvex structure suspended in the eyeball between the aqueous and the vitreous. Its function is to bring rays of light to a focus on retina. Accommodation is produced by variations in the magnitude of this effect.

Levocardia: Displacement of the heart in the extreme left hemithorax.

Lifetime average daily dose (LADD): The total dose received over a lifetime multiplied by the fraction of a lifetime during which exposure occurs, expressed in mg/kg body weight/day.

Lifetime risk: A risk that results from lifetime exposure.

Liquid aerosol: The airborne state of a chemical that is liquid at room temperature and pressure but is nebulized into a particulate atmosphere.

Lordosis: Anterior concavity in the curvature of the cervical and lumbar spine as viewed from the side.

Lowest-observed adverse-effect level (LOAEL): The lowest dose or exposure level of a chemical in a study at which there is a statistically or biologically significant increase in the frequency or severity of an *adverse* effect in the exposed population as compared with an appropriate, unexposed control group.

Lowest-observed effect level (LOEL): In a study, the lowest dose or exposure level of a chemical at which a statistically or biologically significant effect is observed in the exposed population compared with an appropriate unexposed control group. The effect is generally considered not to have an adverse effect on the health and survival of the animal. This term is occasionally misused in place of a LOAEL.

Lung compliance: A measure of the ease in which the lung is extended usually expressed as mL/cmH₂O.

Lung resistance: The total resistance to the movement of air during the expiratory phase of the respiratory cycle expressed as cmH₂O/mL/s.

Macrobrachia: Abnormal size or length of the arm.

Macrodactylia: Excessive size of one or more digits.

Macroglossia: Enlarged tongue, usually protruding.

Macrophage: A bone marrow-derived cell present in the peripheral tissue that serves a wide variety of host defense functions, acting as both nonspecific phagocytes and killer cells, as well as regulators of specific immune reactions. Macrophages have different designations depending on the tissue in which they

are located, such as Kupffer cells in the liver and veiled cells in the lymphatic system.

Macrophthalmia: Enlarged eye(s).

Major histocompatibility complex (MHC): Cell surface molecules that determine tissue compatibility and regulate self-recognition and tolerance. Two major classes are recognized Class I (present on all nucleated cells) and Class II (present on B cells, T cells, and macrophages). MHC molecules direct the course of immune reactivity and are presented in association with antigens by antibody presenting cells (APCs). In humans, MHCs are specifically referred to as human leukocyte antigen (HLA).

Malformation: Defective or abnormal formation; deformity. A permanent structural deviation that generally is incompatible with, or severely detrimental to, normal survival or development.

Malignant: A classification of anticipated biological behavior of neoplasms in which the prognosis for survival is poor. Malignant neoplasms grow rapidly, invade, destroy, and are usually fatal.

Margin of exposure (MOE): The ratio of the NOAEL to the estimated human exposure. The MOE was formerly referred to as the margin of safety (MOS).

Mass median aerodynamic diameter (MMAD): In inhalation toxicology, the median-sized particle based on mass measurement relative to a unit density sphere.

Maximal acceptable toxicant concentration (MATC): In aquatic toxicology, the hypothetical toxic threshold concentration lying in a range bounded at the lower end by the highest tested concentration having no observed effect (NOEC) and at the higher end by the lowest concentration having a statistically significant toxic effect (LOEC) in a life cycle (full chronic) or a partial life cycle (partial chronic) test. This can be represented by NOEC < MATC < LOEC.

Maximum contaminant level (MCL): The maximum level of a contaminant permissible in water as defined by regulations promulgated under the Safe Drinking Water Act.

Maximum daily dose (MDD): Maximum dose received on any given day during a period of exposure generally expressed in mg/kg body weight/day.

Maximum recommended starting dose (MRSD): The highest dose recommended as the initial dose of a drug in a clinical trial. The MRSD is predicted to cause no adverse reactions.

Maximum tolerated dose (MTD): The highest dose of a toxicant that causes toxic effects without causing death during a chronic exposure and that does not decrease the body weight by more than 10%.

Mean corpuscular hemoglobin (MCH): The average amount of hemoglobin per red blood cell.

Mean corpuscular hemoglobin concentration (MCHC): The average hemoglobin concentration per red blood cell.

Mean corpuscular volume (MCV): The average size of the red blood cell.

- Medical device:** Any instrument, apparatus, appliance, material, or other articles, including software, whether used alone or in combination, intended by the manufacturer for use by human beings solely or principally for the purpose of diagnosis, prevention, monitoring, or treatment; alleviation of disease, injury, or handicap; investigation, replacement, or modification of the anatomy or of a physiological process; control of conception; and that which does not achieve its principal intended action of the body by pharmaceutical, immunological, or metabolic means but may be assisted in its function by such means.
- Meiosis:** Cell division occurring in maturation of the sex cell (gametes) by means of which each daughter nucleus receives half the number of chromosomes characteristic of the somatic cells of the species.
- Metaplasia:** The substitution in a given area of one type of fully differentiated cell for the fully differentiated cell type normally present in that area, for example, squamous epithelium replacing ciliated epithelium in the respiratory airways.
- Metastasis:** The dissemination of cells from a primary neoplasm to a noncontiguous site and their growth therein. Metastases arise by dissemination of cells from the primary neoplasm via the vascular or lymphatic system and are an unequivocal hallmark of malignancy.
- Methemoglobin:** Oxidized hemoglobin incapable of carrying oxygen.
- Microcephaly:** Small head.
- Micrognathia:** Shortened lower jaw (mandible); tongue may protrude.
- Micronucleus:** A microscopically detectable particle in a cell that contains nuclear DNA, usually 1/20th to 1/5th the size of the main nucleus. It may be composed of a broken centric or acentric part of a chromosome or a whole chromosome.
- Microphthalmia:** Small eyes.
- Microstomia:** Small mouth opening.
- Microtia:** Small external ear.
- Miotic:** A drug causing pupillary constriction.
- Mitogen:** Mitogens are molecules capable of inducing cellular activation and may include sugars or peptides. The ability of a cell to respond to stimulation with a mitogen (generally assessed by cellular proliferation) is thought to give an indication of the cell's immune responsiveness. Mitogens most commonly employed in immunotoxicology assays include the T cell mitogens concanavalin A (ConA) and phytohemagglutinin (PHA). Mitogens routinely used for assessing B cell proliferation include pokeweed mitogen (PWM) and *Escherichia coli* lipopolysaccharide (LPS).
- Mitogenesis:** The generation of cell division or cell proliferation.
- Mitotic index:** The ratio of the number of cells in a population in various stages of mitosis to the number of cell in the population not in mitosis.
- Mixed lymphocyte response/reaction (MLR):** An in vitro assay that measures the ability of lymphocytes to respond to the presence of allogeneic cells. This proliferation represents the initial stage of the acquisition of cytotoxic T lymphocyte (CTL) function by CD8+ T cells and thus serves as a measure of cell-mediated immunity (CMI). The MLR is a form of lymphoproliferation. Also referred to as mixed lymphocyte culture (MLC).
- Modifying factor (MF):** A factor that is greater than 0 and less than or equal to 10; used in the operational derivation of an RfD. Its magnitude depends upon an assessment of the scientific uncertainties of the toxicological database not explicitly treated with standard UF (e.g., the completeness of the overall database). The default value for the MF is 1. The use of an MF was discontinued by the EPA in 2004.
- Monocardium:** Possessing a heart with only one atrium and one ventricle.
- Mononuclear phagocyte system:** Previously known as the reticuloendothelial system (RES), this system is composed of all phagocytic cells of the body, including monocytes/macrophages and polymorphonuclear cells (i.e., neutrophils).
- Mucosa-associated lymphoid tissue (MALT):** Lymphoid cells and tissues lining the mucosa that serve as the first point of contact with antigen encountered via this route. MALT comprises Peyer's patches, the appendix, tonsils, and lymphoid cells in the lamina propria of the gut.
- Multigravida:** A female pregnant for the second (or more) time.
- Multistage model:** A mathematical function used to extrapolate the probability of incidence of disease from a bioassay in animals using high doses to that expected to be observed at the low doses that are likely to be found in chronic human exposure. This model is commonly used in quantitative carcinogenic risk assessments where the chemical agent is assumed to be a complete carcinogen and the risk is assumed to be proportional to the dose in the low region.
- Mutation:** A structural alteration of DNA that is hereditary and gives rise to an abnormal phenotype. A mutation is always a change in the DNA base sequence and includes substitutions, additions, rearrangements, or deletions of one or more nucleotide bases.
- Mydriatic:** A drug causing pupillary dilatation.
- Myoclonus:** Brief, involuntary twitching of a muscle or a group of muscles.
- Myotonia:** Delayed relaxation of a muscle after an initial contraction.
- Naris (nostril) atresia:** Absence or closure of nares.
- Nasal agenesis:** Absence of the nasal cavity and external nose.
- Natural killer (NK) cells:** A population of lymphocytes distinct from T and B cells, also referred to as large granular lymphocytes (LGLs) because of their

microscopic appearance. NK cells exhibit cytotoxicity against virally infected cells and certain tumor cells. The assessment of NK cell function provides a good measure of innate immunity.

Necrosis: Pathological death of one or more cells or of a portion of tissue or organ, resulting from irreversible damage.

New drug application (NDA): When the sponsor of a new drug believes that enough evidence on the drug's safety and effectiveness has been obtained to meet FDA's requirements for marketing approval, the sponsor submits to FDA an NDA. The application must contain data from specific technical viewpoints for review, including chemistry, pharmacology, medical, biopharmaceutics, and statistics. If the NDA is approved, the product may be marketed in the United States. For internal tracking purposes, all NDAs are assigned an NDA number.

New molecular entity (NME): A novel molecule under development for pharmaceutical purposes. The term encompasses both NCEs, such as small molecule drugs, and new biological entities (NBEs).

Nonocclusive: The site of dermal application of a test substance is open to the air.

Nonthreshold toxicant: An agent considered to produce a toxic effect from any dose; any level of exposure is deemed to involve some risk. Usually only in regard to carcinogenesis.

No-observed adverse-effect level (NOAEL): The highest experimental dose at which there is not statistically or biologically significant increases in frequency or severity of *adverse* health effects, as seen in the exposed population compared with an appropriate, unexposed population. Effects may be produced at this level, but they are not considered to be adverse.

No-observed effect level (NOEL): The highest experimental dose at which there is not statistically or biologically significant increase in the frequency or severity of effects seen in the exposed compared with an appropriate unexposed population.

Normozoospermia: Normal semen sample.

Nose-only exposure: A system for exposing test animals via inhalation in which they are restrained in a tube in such a way that only their nose or snout is exposed directly to the test material.

Nulliparous: A female that never has born viable offspring.

Nystagmus: An involuntary, rapid movement of the eyeball that may be horizontal, vertical, rotatory, or mixed.

Occlusive covering: A bandage or dressing that covers the skin and excludes it from air. Prevents loss of a test substance by evaporation and by increasing tissue penetration.

Occupational exposure limit (OEL): A generic term denoting a variety of values and standards, generally time-weighted average (TWA) concentrations of airborne substances to which a worker can be safely exposed during defined work periods.

Octanol–water partition coefficient (K_{ow}): The ratio of the solubility of a chemical in n-octanol and water at

steady state; also expressed as P . The logarithm of P or K_{ow} (i.e., $\log P$ or K_{ow}) is used as an indication of the propensity of a chemical for bioconcentration by aquatic organisms.

Oligodactyly: Fewer than normal number of digits.

Oligohydramnios (oligoamnios): Reduction in the amount of amniotic fluid.

Oligozoospermia: A subnormal sperm concentration in ejaculate.

Omphalocele: Midline defect in the abdominal wall at the umbilicus, through which the intestines and often other viscera (stomach, spleen, and portions of the liver) protrude. These are always covered by a membranous sac. As a rule, the umbilical cord emerges from the top of the sac.

Oncogene: The activated form of a proto-oncogene. Oncogenes are associated with development of neoplasia.

Optic disk: Ophthalmoscopically visible portion of the optic nerve.

Pachynsis: Abnormal thickening.

Packed cell volume: The percent of blood that contains RBC components; synonymous with hematocrit.

Palpebral: Pertaining to the eyelid.

Pannus: Vascularization and connective-tissue deposition beneath the epithelium of the cornea.

Paresthesia: An abnormal sensation, such as burning, prickling, tickling or tingling.

Patent ductus arteriosus (ductus botalli): An open channel of communication between the main pulmonary artery and the aorta may occur as an isolated abnormality or in combination with other heart defects.

Peripheral blood mononuclear cells (PBMCs)/Peripheral blood mononuclear leukocytes (PBMLs): Leukocytes derived from the peripheral circulation. Because of their accessibility, these cells are often used in *ex vivo* immune function assessment.

Permissible exposure limit (PEL): Similar to an occupational exposure limit (OEL).

Pharmacologically active dose (PAD): The lowest dose tested in an animal species with the intended pharmacological activity.

Photoallergy: An increased reactivity of the skin to UV and/or visible radiation produced by a chemical agent on an immunological basis. Previous allergy sensitized by exposure to the chemical agent and appropriate radiation is necessary. The main role of light in photoallergy appears to be in the conversion of the hapten to a complete allergen.

Photoirritation: Irritation resulting from light-induced molecular changes in the structure of chemicals applied to the skin.

Photosensitization: Sensitization of the skin to UV light, usually due to the action of certain drugs, plants, or other substances; may occur shortly after administration of the substance or may occur only after latent period of days to months. The processes whereby foreign substances, either absorbed locally into the skin or

systemically, may be subjected to photochemical reactions within the skin, either leading to chemically induced photosensitivity reactions or altering the “normal” pathologic effects of light. UV-A is usually responsible for most photosensitivity reactions.

PLA: Product License Application for a biologic.

Plasmid: An autonomously replicating DNA molecule distinct from the normal genome. A plasmid may insert into the host chromosome or form an extra chromosomal element.

Poikilocytosis: Variations in the shape of red blood cells.

Point mutation: Change in the genetic code, usually confined to a single base pair.

Polychromasia: Increased basophilic staining of erythrocytes.

Polycythemia: An increase in the number of red blood cells.

Polydactyly: Extra digits.

Polysomia: A fetal malformation consisting of two or more imperfect and partially fused bodies.

Posterior chamber: Space filled with aqueous anterior to the lens and posterior to the iris of the eye.

Potency: A comparative expression of chemical or drug activity measured in terms of the relationship between the incidence or intensity of a particular effect and the associated dose of a chemical, to a given or implied standard of reference. Can be used for ranking the toxicity of chemicals.

ppb: Parts per billion.

ppm: Parts per million.

Predicate device: A previously marketed medical device that is substantially equivalent to a proposed device. The predicate device is used as a comparison to the proposed device to establish safety and efficacy.

Preneoplastic lesion: A lesion usually indicative that the organism has been exposed to a carcinogen. The presence of preneoplastic lesions indicates that there is enhanced probability for the development of neoplasia in the affected organ. Preneoplastic lesions are believed to have a high propensity to progress to neoplasia.

Prevalence: The percentage of a population that is affected with a particular disease at a given time.

Procarcinogen: An agent that requires bioactivation in order to give rise to a direct acting carcinogen. Without metabolic activation, these agents are not carcinogenic.

Processing aid: A material that contacts a medical device product during the manufacturing process and, therefore, has a potential for affecting product quality and/or may elicit a biological response following the use of a medical device. Solvents, cleaning products, lubricants, and mold-release agents are examples of processing aids.

Progression: Processes associated with the development of an initiated cell to a biologically malignant neoplasm. Sometimes used in a more limited sense to describe the process whereby a neoplasm develops from a benign to a malignant proliferation or from a low-grade to a high-grade malignancy. Progression

is that stage of neoplastic development characterized by demonstrable changes associated with increased growth rate, increased invasiveness, metastases, and alterations in biochemical and morphological characteristics of a neoplasm.

Promoter: *Use in multistage carcinogenesis*—an agent that is not carcinogenic itself but when administered after an initiator of carcinogenesis stimulates the clonal expansion of the initiated cell to produce a neoplasm. *Use in molecular biology*—a DNA sequence that initiates the process of transcription and is located near the beginning of the first exon of a structural gene.

Promotion: The enhancement of neoplasm formation by the administration of a carcinogen followed by an additional agent (promoter) that has no intrinsic carcinogenic activity by itself.

Prothrombin time: A measure of the relative activity of factors in the extrinsic clotting sequence and the common pathway necessary in normal blood coagulation.

Protooncogene: A normal cellular structural gene that, when activated by mutations, amplifications, rearrangements, or viral transduction, functions as an oncogene and is associated with development of neoplasia. Proto-oncogenes regulate functions related to normal growth and differentiation of tissues.

Pseudopregnancy: (a) False pregnancy: condition occurring in animals in which anatomical and physiological changes occur similar to those of pregnancy; (b) the premenstrual stage of the endometrium: so called because it resembles the endometrium just before implantation of the blastocyst.

Ptoxis: Drooping of the upper eyelid.

Pupil: The round opening at the center of the iris that allows transmission of light to the posterior of the eyeball.

Purkinje cells: Modified cardiac muscle cells specialized for conduction of electric excitation from the atrioventricular node through the ventricular septum and throughout the walls of the ventricle. Purkinje cells are organized into fiber bundles that can be readily dissected from the working myocardium and studied in vitro using electrophysiological recording methods.

Purkinje fiber stimulation frequency: The repetition rate for application of brief electric shocks to a Purkinje fiber preparation. A frequency rate of 1 Hz corresponds to 1 shock/s and represents the normal heart rate of 1 beat/s. Frequencies of 0.5 and 2 Hz correspond to bradycardic and tachycardic heart rates.

q1*: The symbol used to denote the 95% upper bound estimate of the linearized slope of the dose–response curve in the low-dose region as determined by the multistage model.

QT interval: The time interval in the electrocardiogram extending from the start of the QRS complex to the end of the T wave and is the measure of the duration of ventricular depolarization and repolarization. The

QRS complex corresponds to the upstroke of the cardiac action potential, and the end of the T wave corresponds to the return of the action potential baseline.

Qualification: For pharmaceuticals, the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or degradation product or a given impurity or degradation profile at the level(s) specified.

Qualification threshold: A limit (%) above which a drug impurity or degradation product should be qualified.

Rachischisis: Absence of vertebral arches in limited area (partial rachischisis) or entirely (rachischisis totalis).

Reference dose (RfD): An estimate (with uncertainty spanning perhaps an order of magnitude or more) of the daily exposure to the human population (including sensitive subpopulations) that is likely to be without deleterious effects during a lifetime. The RfD is reported in units of mg of substance/kg body weight/day for oral exposures or mg/substance/m³ of air breathed for inhalation exposures (RfC).

Regulatory gene: A gene that controls the activity of a structural gene or another regulatory gene. Regulatory genes usually do not undergo transcription into messenger RNA.

Renal hypoplasia: Incomplete development of the kidney.

Reporting threshold: A limit (%) above which a drug impurity or degradation product should be reported.

Reticuloendothelial system (RES): The system composed of all phagocytic cells of the body, including monocytes and tissue macrophages. This system is now more commonly known as the mononuclear phagocytic system.

Resorption: A conceptus that, having implanted in the uterus, subsequently died and is being or has been resorbed.

Resting membrane potential (RMP): In cardiac electrophysiology, RMP is the resting membrane potential in mV and is obtained from the membrane voltage measured immediately before the action potential upstroke. RMP is controlled primarily by inwardly rectifying potassium channels. A decrease in RMP may indicate inhibition of the inward rectifier current.

Reticulocyte: An immature (polychromatic) erythrocyte.

Reticulocytosis: Increased numbers of reticulocytes in the circulation, typically seen in response to regenerative anemia.

Retina: The innermost or nervous tunic of the eye that is derived from the optic cup (the outer layer develops into the complex sensory layer).

Rhinocephaly: A developmental anomaly characterized by the presence of a proboscis-like nose above the eyes, partially or completely fused into one.

Risk: The probability that an adverse effect will occur under a particular condition of exposure.

Risk assessment: The scientific activity of evaluating the toxic properties of a chemical and the conditions of human exposure to it in order both to ascertain the likelihood that exposed humans will be adversely

affected and to characterize the nature of the effects they may experience. May contain some or all of the following four steps:

Hazard identification: The determination of whether a particular chemical is or is not causally linked to particular health effect(s)

Dose-response assessment: The determination of the relation between the magnitude of exposure and the probability of occurrence of the health effects in question

Exposure assessment: The determination of the extent of human exposure

Risk characterization: The description of the nature and often the magnitude of human risk, including attendant uncertainty

Risk management: The decision-making process that uses the results of risk assessment to produce a decision about environmental action. Risk management includes the consideration of technical, scientific, social, economic, and political information.

Rudimentary rib: Imperfectly developed riblike structure.

Safety pharmacology: The investigation of the pharmacological effects of a drug other than the desired primary therapeutic effect. Safety pharmacology studies are focused on identifying adverse effects on physiological functions.

Schistoglossia: Cleft tongue.

Sclera: The white tough covering of the eye that, with the cornea, forms the external protective coat of the eye.

Seminiferous epithelium: The normal cellular components within the seminiferous tubule consisting of Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids.

Semiocclusive: The site of dermal application of test substance is covered; however, the movement of air through covering is not restricted.

Sensitization (allergic contact dermatitis): An immunologically mediated cutaneous reaction to a substance.

Septal agenesis: Absence of nasal septum.

Sertoli cells: Cells in the testicular tubules providing support, protection, and nutrition for the spermatids.

Short-term exposure limit (STEL): A time-weighted occupational exposure limit that the ACGIH indicates should not be exceeded any time during the work day. Exposures at the STEL should not be longer than 15 min and should not be repeated more than four times per day. There should be at least 60 min between successive exposures at the STEL.

Sister chromatid exchange: The morphological reflection of an interchange between DNA molecules at homologous loci within a replicating chromosome.

Skin immune system (SIS): Cells associated with the skin that participate in immunity. Includes Langerhans cells, dendritic cells, and keratinocytes. Alternatively known as SALT.

Slope factor: See Cancer potency factor (CPF).

Smoke: The airborne state of a chemical that is combusted and allowed to condense into a particulate matter.

SNUR: Significant new use rule.

Somatic cell: A normal diploid cell of an organism as opposed to a germ cell, which is haploid. Most neoplasms are believed to begin when a somatic cell is mutated.

Sorbitol dehydrogenase (SDH): An enzyme of liver origin, whose blood concentration rises in response to hepatocellular injury.

Spermatocytogenesis: The first stage of spermatogenesis in which spermatogonia develop into spermatocytes and then into spermatids.

Spermiation: The second stage of spermatogenesis in which the spermatids transform into spermatozoa.

Spina bifida: Defect in closure of bony spinal cavity.

Standardized mortality ratio: The number of deaths, either total or cause specific, in a given group expressed as a percentage of the number of deaths that could have been expected if the group has the same age- and sex-specific rates as the general population. Used in epidemiologic studies to adjust mortality rates to a common standard so that comparisons can be made among groups.

STEL: See *Short-term exposure limit (STEL)*.

Stereotypy: The constant repetition of gestures or movements that appear to be excessive or purposeless.

Superficial sloughing: Characterized by dead tissue separated from a living structure. Any outer layer or covering that is shed. Necrosed tissue separated from the living structure.

Surface area scaling factor: The intra- and interspecies scaling factor most commonly used for cancer risk assessment by the US EPA to convert an animal dose to an HED: milligrams per square meter surface area per day. Body surface area is proportional to basal metabolic rate; the ratio of surface area to metabolic rate tends to be constant from one species to another. Since body surface area is approximately proportional to an animal's body weight to the $2/3$ power, the scaling factor can be reduced to milligrams per body weight $^{2/3}$.

Sympodia: Fusion of the lower extremities.

Syndactyly: Partially or entirely fused digits.

T cell/T lymphocyte: Lymphocytes that recognize specific antigens via a complex of molecules known collectively as the T-cell antigen receptor (TCR). T cells are primarily responsible for the induction and maintenance of cell-mediated immunity (CMI), although they also regulate humoral-mediated immunity (HMI) and some nonimmune effector mechanisms. A variety of T cell populations exist, including helper T cells, cytotoxic T cells, inducer T cells, and regulatory T cells. T cells mature in the thymus.

TC_{Lo} (toxic concentration low): The lowest concentration of a substance in air required to cause a toxic effect in some of the exposed population.

TD_{Lo} (toxic dose low): The lowest dose of a substance required to cause a toxic effect in some of the exposed population.

Tetralogy of Fallot: An abnormality of the heart that includes pulmonary stenosis, ventricular septal defect, dextroposition of the aorta overriding the ventricular septum and receiving blood from both ventricles, and right ventricular hypertrophy.

Teratozoospermia: Fewer than 30% spermatozoa with normal morphology.

Thoracogastroschisis: Midline fissure in the thorax and abdomen.

Threshold limit value (TLV): The TWA concentration of a substance below which no adverse health effects are expected to occur for workers assuming exposure for 8 h per day, 40 h per week. TLVs are published by the American Conference of Governmental Industrial Hygienists (ACGIH).

Time-weighted average (TWA): An approach to calculating the average exposure over a specified time period.

TL_m or TL₅₀ (median tolerance limit): In aquatic toxicology, the concentration of material in water at which 50% of the test organisms survive after a specified time of exposure. The TL_m (or TL₅₀) is usually expressed as a time-dependent value (e.g., 24 h or 96 h TL₅₀).

Tonic convulsion: A convulsion in which muscle contraction is sustained.

Totalis or partialis: Total or partial transposition of viscera (due to incomplete rotation) to the other side of the body; heart most commonly affected (dextrocardia).

Tracheal stenosis: Constriction or narrowing of the tracheal lumen.

Transformation: Typically refers to tissue culture systems where there is conversion of normal cells into cells with altered phenotypes and growth properties. If such cells are shown to produce invasive neoplasms in animals, malignant transformation is considered to have occurred.

Triglycerides: Synthesized primarily in the liver and intestine; the major form of lipid storage.

Ulceration: The development of an inflammatory, often suppurating lesion, on the skin or an internal mucous surface of the body caused by superficial loss of tissue, resulting in necrosis of the tissue.

Ultimate carcinogen: That form of the carcinogen that actually interacts with cellular constituents to cause the neoplastic transformation. The final product of metabolism of the procarcinogen.

Uncertainty factor (UF): One of several, generally 10-fold factors, applied to an NOAEL or an LOAEL to derive an RfD from experimental data. UFs are intended to account for (a) the variation in the sensitivity among the members of the human population,

(b) the uncertainty in extrapolating animal data to human, (c) the uncertainty in extrapolating from data obtained in a less-than-lifetime exposure study to chronic exposure, (d) the uncertainty in using an LOAEL rather than an NOAEL for estimating the threshold region, and (e) uncertainty with extrapolation when the database is incomplete.

Unilobular lung: In the rat fetus, a condition in which the right lung consists of one lobe instead of four separate lobes.

Unit cancer risk: A measure of the probability of an individual's developing cancer as a result of exposure to a specified unit ambient concentration. For example, an inhalation unit cancer risk of 3.0×10^{-4} near a point source implies that if 10,000 people breathe a given concentration of a carcinogenic agent (e.g., $1 \mu\text{g}/\text{m}^3$) for 70 years, 3 of the 10,000 will develop cancer as a result of this exposure. In water, the exposure unit is usually $1 \mu\text{g}/\text{L}$, whereas in air it is $1 \mu\text{g}/\text{m}^3$.

UDS: Unscheduled DNA synthesis that occurs at some stage in the cell cycle other than S-phase in response to DNA damage and is usually associated with DNA excision repair.

Upper 95% confidence limit: Assuming random and normal distribution, this is the range of values below which a value will fall 95% of the time.

Upper bound cancer risk assessment: A qualifying statement indicating that the cancer risk estimate is not a true value in that the dose-response modeling used provides a value that is not likely to be an underestimate of the true value. The true value may be lower than the upper bound cancer risk estimate, and it may even be close to zero. This results from the use of a statistical upper confidence limit and from the use of conservative assumptions in deriving the cancer risk estimate.

Urea nitrogen (BUN): The end product of protein catabolism. Blood levels can rise after renal (glomerular) injury.

USP negative control plastic RS: A standardized plastic produced by the USP for use as a control material in some biocompatibility assays.

Vaginal plug: A mass of coagulated semen that forms in the vagina of animals after coitus; also called copulation plug or bouchon vaginal.

Vapor: The airborne state of a chemical that is liquid at room temperature and pressure but is volatile.

Variation: In developmental toxicology, an alteration that represents a retardation in development, a transitory alteration, or a permanent alteration not believed to adversely affect survival, growth, development, or functional competence.

Vitreous: Transparent, colorless, and mass of soft, gelatinous material filling the space in the eyeball posterior to the lens and anterior to the retina.

V_{max} : In cardiac electrophysiology, this is the maximum rate of depolarization measured in Volts/seconds (V/s)

obtained by taking the first derivative of the rising phase of the action potential. V_{max} amplitude is determined primarily by the activity of cardiac sodium channels. A decrease in V_{max} indicates sodium channel blockade and corresponds to a broadening of the electrographic QRS interval and, potentially, a slowing of conduction velocity in the intact heart.

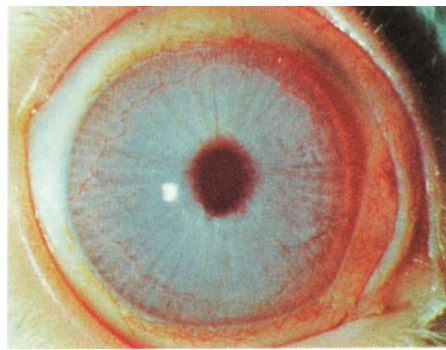
Voluntary risk: Risk that an individual has consciously decided to accept.

Whole-body exposure: A system for exposing test animals via inhalation in which they are placed in a chamber in such a way that their entire bodies are exposed directly to the test material.

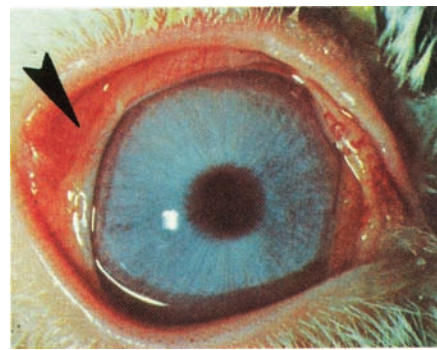
Xenobiotic: Any substance that is foreign to the immune system; in the context of immunotoxicology, the term generally refers to nonbiological chemicals or drugs.

REFERENCES

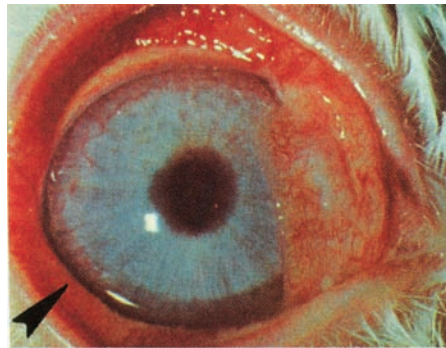
- Balls, M., Blaauboer, B., Brusick, D., Frazier, J., Lamb, D., Pemberton, M., Reinhart, C., Roberfroid, M., Rosenkrantz, H., Schmid, B., Spielmann, H., Stamatii, A.-L., and Walum, E. (1990) *Report and Recommendations of the CAA/ERGATT Workshop on the Validation of Toxicity Test Procedures*, ALTA 18,313.
- Cronin, E. (1980) *Contact Dermatitis*, Churchill Livingstone, New York, Chapters 1–17.
- Environ Corporation (1980) *Risk Assessment Guidance Manual*, AlliedSignal, Inc., Morristown, NJ.
- Hallenbeck, W.G. and Cunningham, K.M., eds. (1986) *Quantitative Risk Assessment for Environmental and Occupational Health*, Lewis Publishers, Chelsea, MI, Appendix 2.
- Klaassen, C.D., Amdur, M.O., and Doull, J., eds. (1991) *Casarett and Doull's Toxicology, The Basic Science of Poisons*, 4th edn., Pergamon Press, New York.
- Maronpot, R.R. (1991) *Handbook of Toxicologic Pathology*, Academic Press, San Diego, CA, pp. 127–129.
- Marzulli, F.N. and Maibach, H.I., eds. (1977) *Dermatotoxicology*, 2nd edn., Hemisphere Publishing Corporation, Washington, DC.
- Middle Atlantic Reproduction and Teratology Association (1989) *A Compilation of Terms Used in Developmental Toxicity Evaluations*.
- Morris, W., ed. (1978) *The American Heritage Dictionary of the English Language*, New College edition, Houghton Mifflin Company, Boston, MA.
- Rand, G.M., ed. (1995) *Fundamentals of Aquatic Toxicology*, 2nd edn., Taylor & Francis Group, Washington, DC.
- Stedman's Medical Dictionary*, 25th edn., Williams & Wilkins, Baltimore, MD, 1990.
- United States Environmental Protection Agency (1984) *Federal Insecticide, Fungicide, Rodenticide Act, Pesticide Assessment Guidelines*, Hazard Evaluation Division, Guidance for Evaluation of Dermal Sensitization.
- United States Environmental Protection Agency (1989) *Glossary of Terms Related to Health, Exposure and Risk Assessment*, Air Risk Information Support Center, EPA No.450/3-88/016.
- United States Food and Drug Administration (2007) www.fda.gov



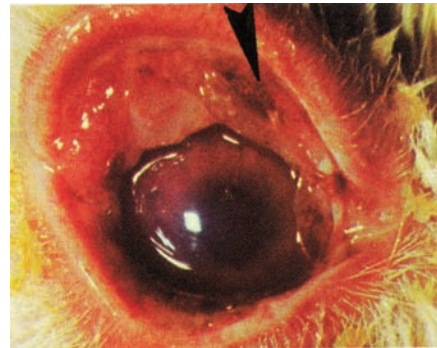
Normal eye



1 Redness

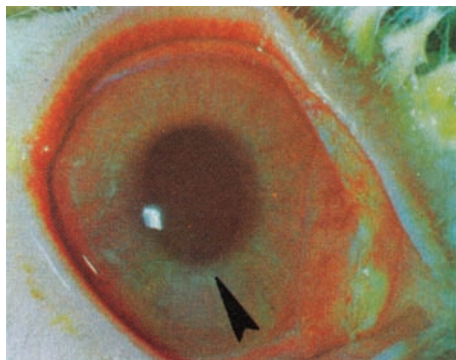


2 Redness

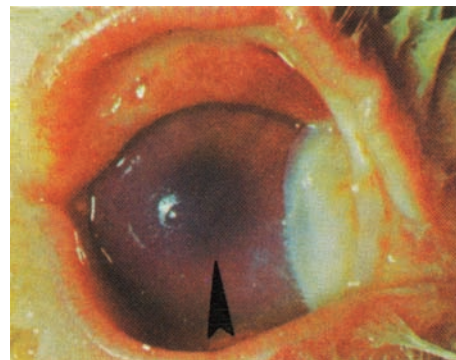


3 Redness

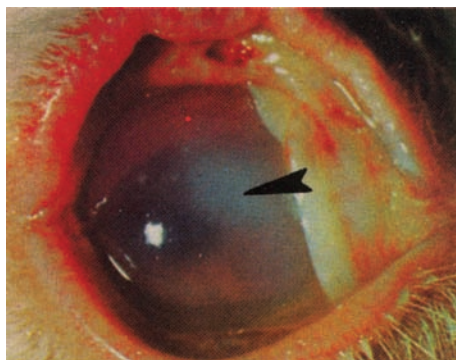
FIGURE 4.6 Photographs demonstrating the four grades for conjunctival redness. Conjunctival redness is typically not homogeneous; therefore, only the most severely affected area of the conjunctiva should be graded as shown by the arrows in the photographs.



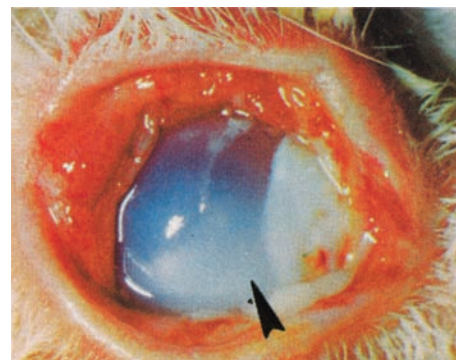
1 Opacity



2 Opacity

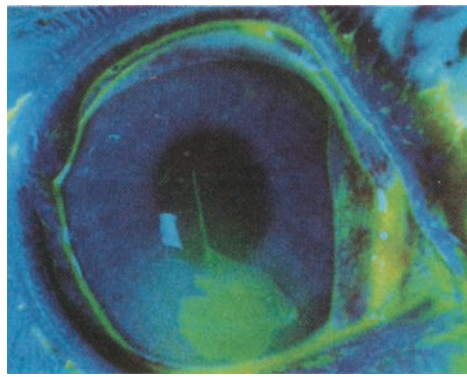


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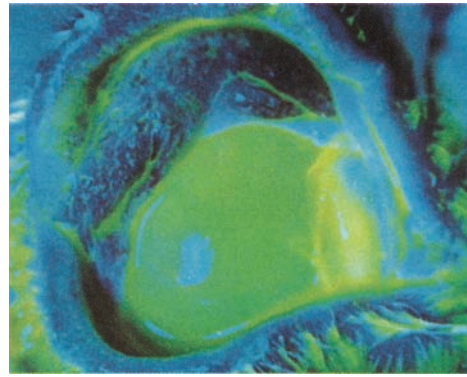


4 Opacity

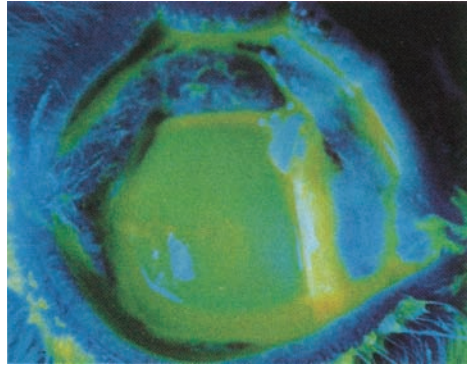
FIGURE 4.7 Photographs demonstrating the four grades for corneal opacity. Because a corneal lesion is not distributed homogeneously, the most severely affected part of the cornea (see arrows) is graded.



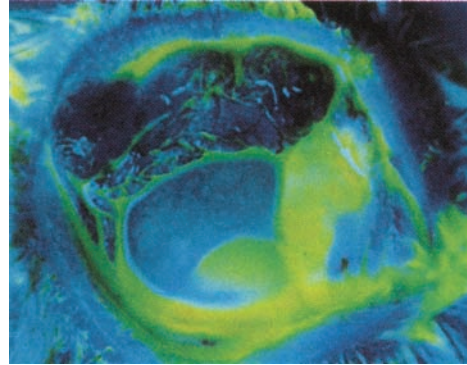
1 Opacity



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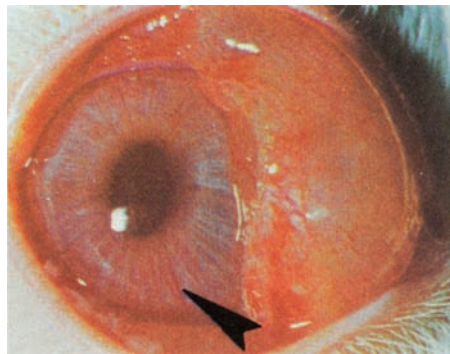


3 Opacity

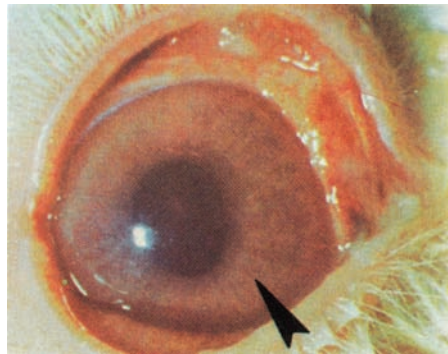


4 Opacity

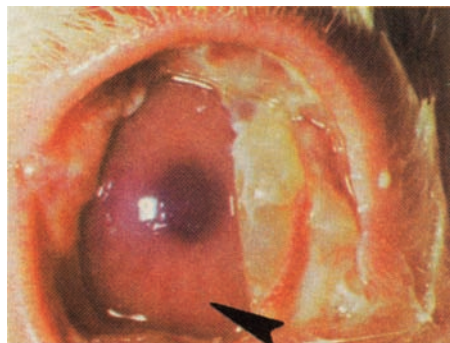
FIGURE 4.8 Eyes stained with fluorescein and photographed under UV light. Flourescein-stained areas demarcate corneal erosion, not opacity. Therefore, the areas stained with flourescein do not necessarily correspond to the grades for opacity.



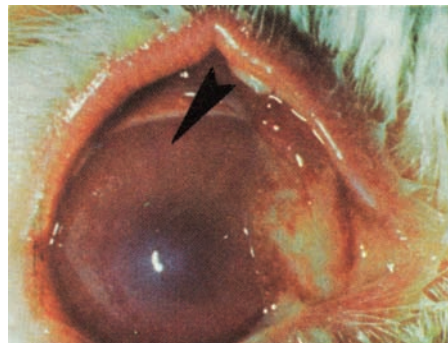
1 Iritis



1 Iritis



2 Iritis



2 Iritis

FIGURE 4.9 Two grades for iritis demonstrated. Grade 1 is a deepening of iridial rugae, or injection (hyperemia) of iridial vessels. The upper photograph clearly shows injection of the secondary vessels of the iris, but this finding is more difficult to perceive in the upper right photograph due to loss of corneal clarity. Grade 2 iritis involves no reaction to light, hemorrhage, and/or destruction of the iris. Since this is almost invariably accompanied by significant corneal opacity, it may be difficult to observe. The two lower photographs demonstrate hemorrhage of the iris.

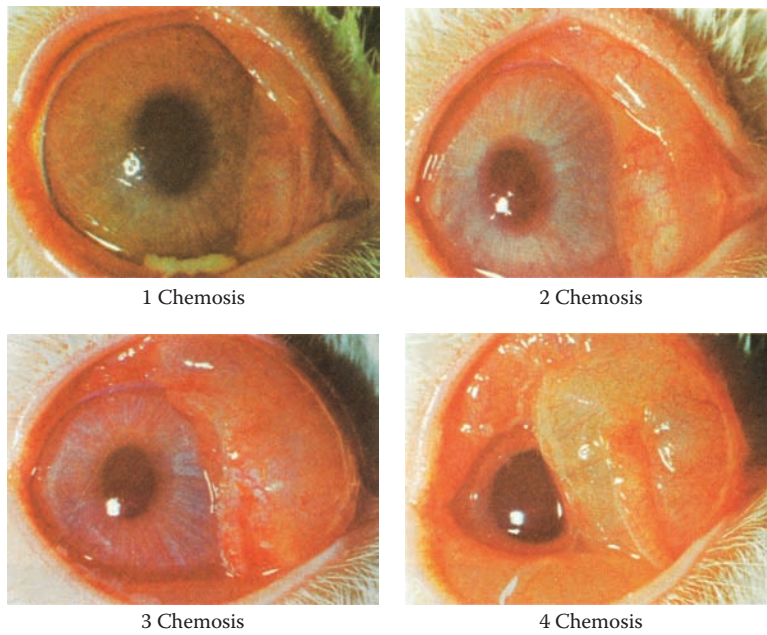


FIGURE 4.10 Photographs intended to indicate the degree of difference between each conjunctival grade. They may not accurately represent chemosis because the eyes have been held open to show other aspects of irritation.

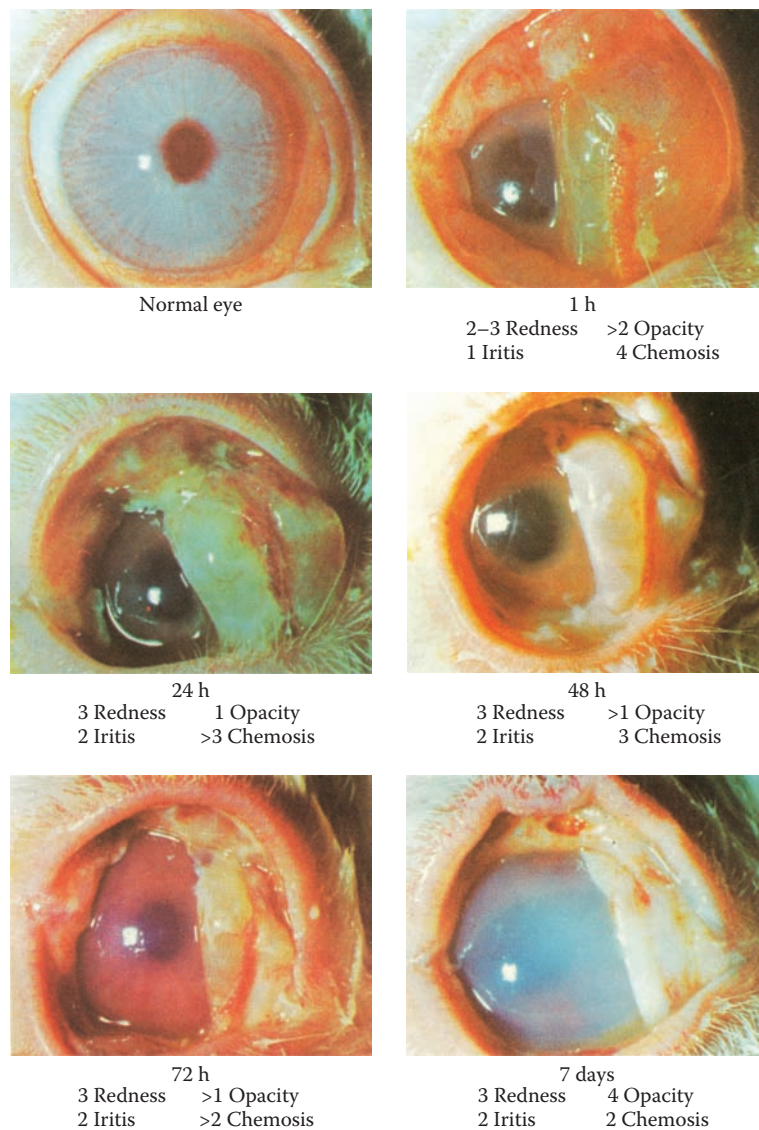


FIGURE 4.11 A time sequence of the same eye shown from before administration of an irritant to 7 days after exposure.

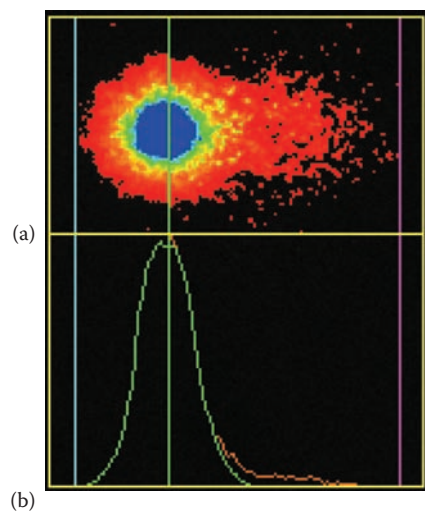


FIGURE 13.1 (a) Image of genomic DNA from single cell used for Comet analysis; high molecular weight DNA remains in head on left and lower molecular weight DNA fragments migrate to right. Blue line on left marks start of genomic DNA, green line marks center of genomic DNA, and purple line on right marks furthest migration of DNA fragments. (b) Graphic representation of DNA density permitting quantification of DNA migration with genomic DNA in area covered by green curve and DNA fragments under the red curve.

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